ESSENTIALS

OF

PRACTICAL BACTERIOLOGY

CURTIS

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To Christopher Heath Esq., LL.D. (Hon. Causa).,
Past President of the Royal College of Surgeons of
England.

With the Author's compliments & kind regards.

Nov. 1900.

PRACTICAL BACTERIOLOGY

By the same Author.

Translated and Edited from the French.

MASTOID ABSCESSES AND THEIR TREATMENT. By Prof. A. Broca, M.D., and F. Lubet-Barbon, M.D. With Coloured Illustrations, crown 8vo. 6s.

'Mr. Curtis has done well in introducing to English surgeons the valuable memoir by Broca and Lubet-Barbon, a work too little known in this country.'

The Lancet.

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THE ESSENTIALS

OF

PRACTICAL BACTERIOLOGY

AN ELEMENTARY LABORATORY BOOK FOR
STUDENTS AND PRACTITIONERS

BY

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TO

THE MEMORY OF

A FRIEND

PREFACE

The lessons now published, in somewhat expanded form, were originally given in the years 1896 and 1897, when the writer had the great good fortune to be in charge of the Bacteriological Department at University College, London, under, successively, Professor Victor Horsley, F.R.S., and Professor Sidney Martin, F.R.S.

The course of study is such as is generally required by those working for a Diploma in Public Health; but attention has been paid to the special needs of clinical bacteriologists. As far as possible, only the simplest and most reliable methods of staining are mentioned.

Minute details of technique have been mentioned in order to facilitate the work of those who may not have recently passed through a class of Morbid Histology. It is hoped that the descriptions will prove sufficiently clear, so that the student may be able to carry out the directions without having constantly to appeal to the teacher, who, otherwise—and almost more so than in any similar subject—needs to be continuously at the side of the beginner, a feat not easy to accomplish in a large class.

In the text, descriptions of the cultivations precede those of the microscopic appearances, as newly made cultures, after incubation over-night, should invariably be inspected before commencing another day's work, and laboratory cultures are available from the first for examination by the student, in the intervals of preparing the film specimens &c. A good plan is to have a stock of typical cultures always at hand, sterilised by formalin, as directed on p. 33.

Objection may be taken to the amount of detail given in dealing with certain subjects, notably ringworm, and cancer. These exceptions to the general rule are meant only for advanced students; and the intention is to provide a starting-point for research, in the directions indicated, by the application of modifications of methods and media already known.

The methods adopted by Plimmer in his recently recorded brilliant investigations on cancer are given in detail, so that this work may be repeated, the results confirmed, or, if need be, qualified; and then still further extended. The repetition of a recorded experiment often leads to the most valuable discoveries; and the establishment of a *single new fact* in the ætiology of a disease may confer untold benefit upon suffering humanity.

As regards the illustrations, the majority have been prepared expressly for this work, and, where not otherwise stated, are from the writer's specimens; and the illustrators, Mrs. Danielsson, and Mr. Sheills, of the firm of Messrs. John Bale, Sons, and Danielsson, Ltd., deserve the highest praise for the great pains they have taken, and the skill they have exhibited, in carrying out their difficult task.

For the loan of blocks of most of the apparatus I have to thank Messrs. Baird and Tatlock, Messrs. Swift and Son kindly providing the two illustrating micrometers, and Messrs. R. and J. Beck the two showing their new centrifuge.

Several illustrations have been borrowed from sources mentioned in the text, and for the loan of these I have to express my great obligations to the respective authors and publishers.

It has often been necessary to quote freely from current English text books; and though, as far as possible, such sources have been duly acknowledged in the text, it will be convenient to mention the most important of these here, as, together with Sternberg's classical work, they will form a useful reference library for the student. Abbott's 'Principles of Bacteriology,' Crookshank's 'Bacteriology and Infective Disease,' Frankland's 'Micro-organisms in Water,' Hewlett's 'Manual of Bacteriology,' Kanthack and Drysdale's 'Practical Bacteriology,' Muir and Ritchie's 'Manual of Bacteriology,' Sims Woodhead's 'Bacteria and their Products,' Schenk's 'Manual of Bacteriology,' Symmers's Translation of Thoinot, and Masselin's 'Outlines of Bacteriology.'

It remains my pleasing duty to gratefully recognise the extreme patience and invariable courtesy shown, on every possible occasion, by both Publishers and Printers alike.

H. J. CURTIS.

HARLEY STREET, W. *October* 1900.

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Corrigenda

Pages 70 and 71, *for* **Penicilium** *read* **Penicillium**

Page 123, line 31, *for* Septic thromboses *read* Septic thrombosis

Pages 53e, 55, 57, and 61, for *Saccharomyces cerivisiæ* read *Saccharomyces cerevisiæ*.

THE ESSENTIALS
OF
PRACTICAL BACTERIOLOGY

PART I

MANUFACTURE OF THE NUTRIENT MEDIA, AND GENERAL TECHNIQUE

Sterilisation of glass apparatus.—All flasks, petri-dishes, and other glass apparatus must be carefully sterilised before use; and here attention must be drawn to the fact that the packing materials used by the manufacturers are teeming with organisms and their spores, particularly the *Bacillus subtilis,* or hay bacillus, the spores of which resist the action of steam for prolonged periods.

New glass apparatus is cleaned as follows:

1. All obvious dirt is cleaned away by the free use of tap water, bottle brush, and, if necessary, sand.

2. Commercial nitric or hydrochloric acid is then employed to cleanse still further, and to kill the resistent spores.

3. The acid is removed by the free use of tap water, and the flasks inverted to drain. They may be allowed to drain in this way in the hot-air chamber at 100° C.

4. They are then plugged with wool. Flasks and wool are now sterilised for three hours at 150° C. in the hot-air steriliser.

5. When the steriliser has been allowed to cool for twenty minutes

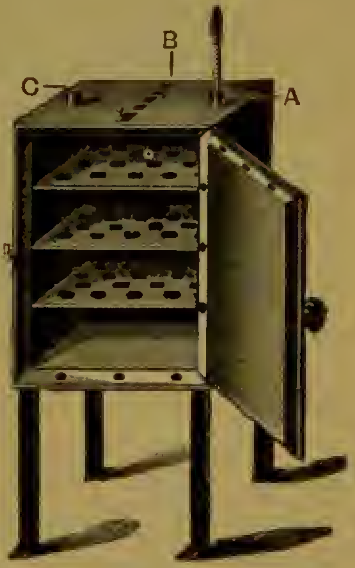

FIG. 1.—HOT-AIR STERILISER.
A, thermometer; B, apertures which can be closed by a shutter or sliding door; C, gas regulator.

or so, the plugged flasks are transferred to a cupboard with glass doors, and stocked for use.

In the case of petri-dishes, after having been cleaned &c., as directed, they are wrapped in 'German blotting-paper,' and sterilised for three hours at 150° C., in the same way as the flasks. They are then stored ready for use in the glass cupboard.

LESSON I

NUTRIENT MEDIA

Nutrient broth.—1. To a litre of tap water in an enamelled saucepan or sterile litre flask, add Liebig's Extract, 5 grammes;[1] Witte's peptone, 10 grammes; sodium chloride, 5 grammes.

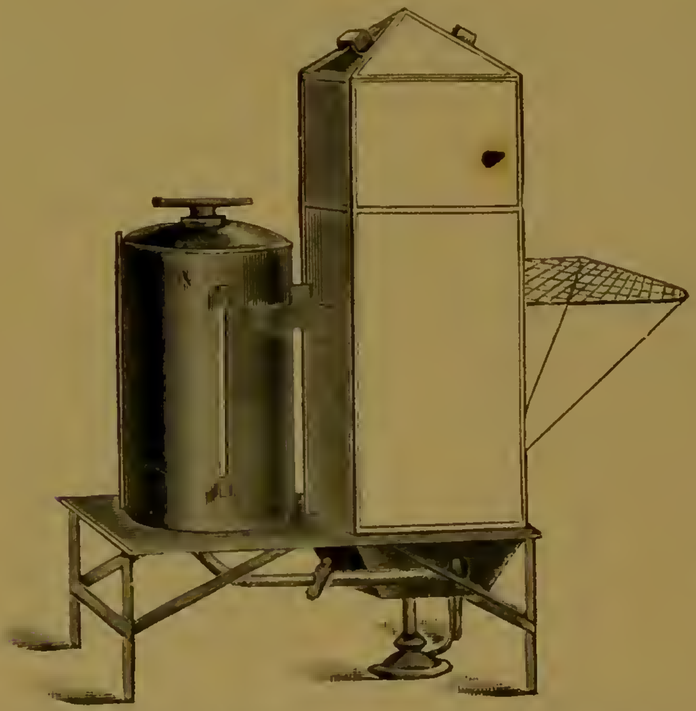

FIG. 2.—STEAM STERILISER, OR 'STEAMER,' WITH REMOVABLE UPPER CHAMBER, AND SUPPLYING CISTERN OF WATER ATTACHED. (Exterior)

2. Mix, stirring up the contents of the saucepan with a glass rod.

3. Boil over Bunsen flame (or in steam steriliser, if litre flask used).

4. Neutralise with a 1 per cent. solution of sodium carbonate; then make slightly alkaline. Filter into a sterile litre flask.

5. Place in steamer (figs. 2 and 3) for half to three-quarters of an hour. The albumen is coagulated, and the fluid portion should be of a clear straw colour.

6. Fold a large filter-paper (in the manner indicated on p. 5),

---

[1] Smear with spatula on to a piece of paper, a similar-sized piece of paper being used as a counterpoise in the other scale-pan; weigh, and then put paper and extract into the saucepan.

place in glass filter, moisten it with water, and filter off the coagulated albumen &c.

7. From the litre of nutrient broth thus obtained, 300 c.c. are poured into each of two sterile flasks of 600 c.c. capacity, which are plugged with sterile wool.

(*a*) *From the first* of these two, nutrient gelatine is made according to directions, p. 7.

(*b*) *From the second*, nutrient agar-agar is made according to directions, p. 9.

(*c*) The broth remaining in the litre flask (about 400 c.c.) is poured into sterile test-tubes, about 6 c.c. to each tube, the plug replaced, and the tubes then sterilised in the steamer for ten to fifteen minutes on three successive days (see p. 4).

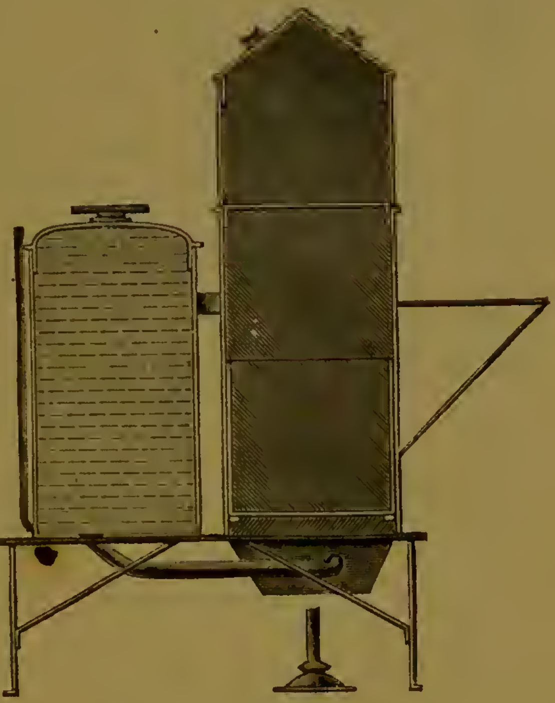

FIG. 3.—STEAM STERILISER SEEN IN SECTION

N.B. If, for any reason, the broth is not made into nutrient gelatine &c., or poured into test-tubes at once, it must be sterilised in the original flask on three successive days. It may then be kept in stock till required.

If the whole litre of broth is to be used as such, none being wanted for making nutrient gelatine or agar, it may be at once poured into the test-tubes and sterilised three times, as above.

The foregoing method, in which Liebig's Extract is employed, is quick, cheap, and perfectly reliable for ordinary purposes.

In the older methods the best meat—beef, veal, &c.—is employed, and doubtless a more nutritious medium is thereby obtained. It is, however, a longer and considerably more expensive method than the former, and the broth resulting can hardly be of so uniform a quality each time as when Liebig's Extract is used.

The older method is as follows:

1. A pound of beef (veal, &c.), as free from fat as possible, is passed through a mincing machine, and then placed in one litre of cold distilled water, allowing the mixture to stand in a cold place overnight.

2. After twenty-four hours the whole mass is strained through a cloth[1] into a litre flask, as much fluid as possible being squeezed through; and distilled water added, if necessary, to make the volume again up to one litre.

3. To this litre of fluid are added peptone, 10 grammes; sodium chloride, 5 grammes.

4. The fluid is thoroughly boiled in the steam steriliser.

5. Neutralise carefully with a 1 per cent. solution of sodium carbonate, then make slightly alkaline.

6. Boil again in steamer for half to three-quarters of an hour.

7. Filter through a folded moistened filter-paper into litre flask or test-tubes. Plug with wool.

8. Sterilise the filtrate in steamer on three successive days, for ten to fifteen minutes each day.

The object of sterilising the different media on three successive days.—By the first day's exposure to a temperature of 100° C. the bacilli actually present are destroyed. Spores, such as those of the hay bacillus (*Bacillus subtilis*), are also present, and may not be killed by this exposure to 100° C.

Most of the spores, however, develop into bacilli by the second day, so that the second sterilisation is effective. As there may be present spores which require more than one day for their development into bacilli, the sterilisation must be repeated on the third day, after which the broth may be safely assumed to be sterile.[2] If these precautions have not been taken, or are insufficiently carried out, the spores will develop into bacilli, and contamination with the latter will be indicated by a turbidity of the previously clear straw-colour fluid.

For special purposes, to ordinary nutrient broth, glycerine, sugar, phenol, formate of sodium, &c. may be added.

Glycerine broth.—5 to 8 c.c. of glycerine are added to every 100 c.c. broth, before the final sterilisation. This is especially useful, for example, for the culture of the *Bacillus tuberculosis* in a fluid medium.

Sugar broth (Glucose broth).—To every 100 c.c. nutrient broth, 1 or 2 grammes of grape sugar (glucose) are added, after neutralisation.

In practice, 1 per cent. sugar broth is found to be better than

---

[1] The thin muslin-like material used commercially for wrapping up butter, and known as 'butter-cloth,' or '*butter-muslin*,' is useful for this purpose. It should be sterilised and kept in large, wide-mouthed, glass-stoppered bottles, ready for use.

[2] Hewlett (*Manual of Bacteriology*) attributes the sterilising effect of repeated heatings partly to the injurious action of alternate heating and cooling.

2 per cent. for anaërobes (such as those causing tetanus, malignant œdema, &c.), and it is chiefly employed for their cultivation, and also for demonstrating the formation of gas by certain organisms, e.g. *B. coli*.

Formate of sodium broth.—Formate of sodium, in the proportion of 0·5 gramme to every 100 c.c., is added to nutrient broth before the final sterilisation of the medium. This is used for the cultivation of anaërobes.

Phenol broth.—Pure phenol is added in various proportions to nutrient broth after the final sterilisation. This medium has been largely used in the attempt to separate *B. typhosus* and *B. coli* from a mixture containing other organisms; and in the proportion of $\frac{1}{10}$ c.c. of a 5 per cent. solution carbolic acid to 10 c.c. of nutrient broth—which means a percentage of 0·05 phenol—the growth of other non-pathogenic organisms is certainly checked, whilst the two specially mentioned above appear to grow uninterruptedly. It is, of course, useless as a method of separating *B. typhosus* from *B. coli*, the more resistent organism; and the danger of killing the typhoid, whilst the coli organism still continues to develop, has largely limited the use of this medium.

'**Parietti broth**' is also used in the attempt to separate *B. typhosus* and *B. coli* from a mixture of other less resistent organisms. It is made by adding to tubes containing 10 c.c. neutral nutrient broth, varying quantities—from 3 to 9 drops of the following solution: Pure phenol, 5 grammes; pure hydrochloric acid, 4 grammes; distilled water, 100 c.c.

The amount used for each tube is noted; and after keeping them at 37° C. for twenty-four hours, so that any contaminating organisms which may have fallen into the tubes at the time of adding the mixture may be destroyed, the medium is ready for use.

Various other substances have been added to nutrient broth for special purposes, besides those mentioned already, but the latter are perhaps the best known and most generally useful.

To fold a filter-paper.—The semicircle A D B (fig. 4) represents a circular filter-paper folded in two along the diameter A B. The paper is again folded by bringing the point A over the point B, so as to form a crease, C D, separating the quadrants A D C, B D C. A series of segments separated by creases are similarly made as follows. Fold the paper so as to bring the point A over D, producing the crease E C; bring B to D, making a crease F C. Bring A to F, making a crease G C; and B to E, making a crease H C. Then bring A to E and B to F. All the creases are on the upper surface of the semicircular piece of paper. Turn the under surface uppermost, and subdivide each segment, A C I, I C E, &c., in the same way, the creases now made being on the side opposite to those which were first made. When this has been completed, the filter-paper, when opened up (fig. 5), will present a zigzag margin, *a b c d e*; but at one place the zigzag is replaced by a 'bay,' *e f g h*, and a similar one will be found on examining the margin of the filter-paper immediately opposite the first seen. Each of these is to be subdivided by a crease, which divides into two equal parts the segment indicated in the figure by the line *f g*, so as to form a zigzag (*e f* * *g h*) instead of a 'bay' (*e f g h*).

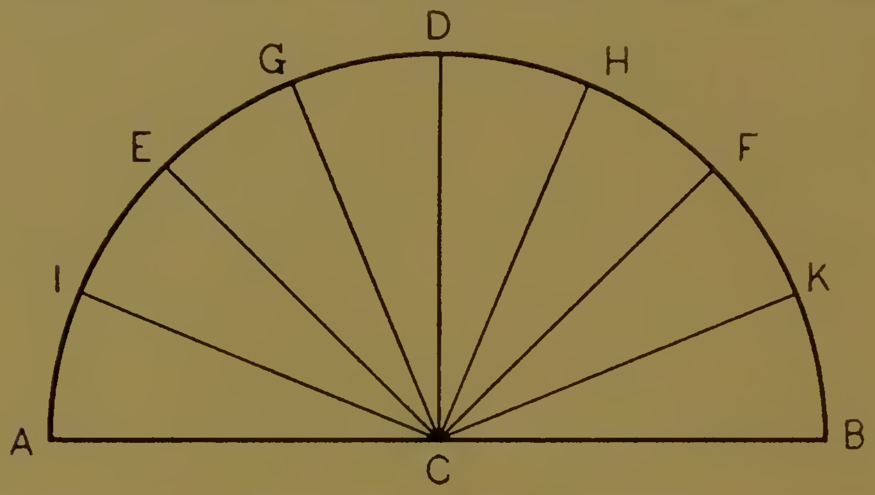

Fig. 4.—DIAGRAM OF FOLDED FILTER-PAPER

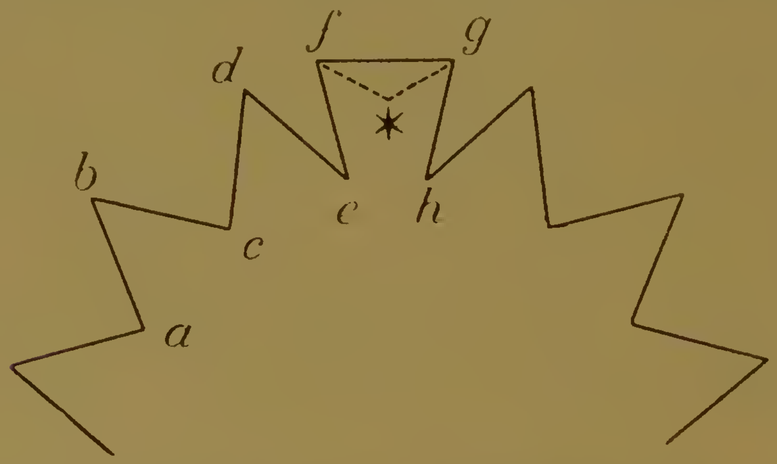

Fig. 5.—DIAGRAM OF EDGE OF OPENED-OUT FILTER-PAPER TO SHOW THE 'BAY' (*e f g h*) AND ZIGZAG (*e f* * *g h*) REFERRED TO IN TEXT

The paper, when thus completely folded, is placed in the glass filter and moistened with distilled water before use.

The object of folding the paper is to increase the area available for filtration; at the same time, less paper comes into close contact with the glass filter than if no ridges were present. Filtration is thus hastened for both reasons, and is further accelerated by moistening the paper, especially in the case of nutrient gelatine and nutrient agar, where rapid filtration is a *sine qua non*. The last-mentioned media have a tendency to clog the pores of the paper, unless this is previously moistened. Even with this precaution, nutrient agar presents serious difficulty when filtration is attempted, even if a hot-water jacket (fig. 6) surrounds the filter.

LESSON II (A)

NUTRIENT MEDIA (*continued*)

Nutrient gelatine.—1. To one of the two 600 c.c. flasks, containing 300 c.c. nutrient broth set aside from the last lesson, add 12½ per cent.[1] (i.e. 37½ grammes) of Hesteberg's 'gold label' gelatine.

2. Place in steamer for one hour, to thoroughly dissolve the gelatine.

3. Neutralise again with 1 per cent. sodium carbonate solution, as the gelatine added to the slightly alkaline broth makes it acid. There may be considerable effervescence.

4. Clarify, by adding the white of an egg.

5. Boil in steamer for a quarter of an hour.

6. Filter through a moistened folded filter-paper, placed in a hot-water filter (fig. 6), into a fresh flask. The filtrate should be quite clear and translucent; if not, filter once more through butter-cloth, and then through a fresh filter-paper.

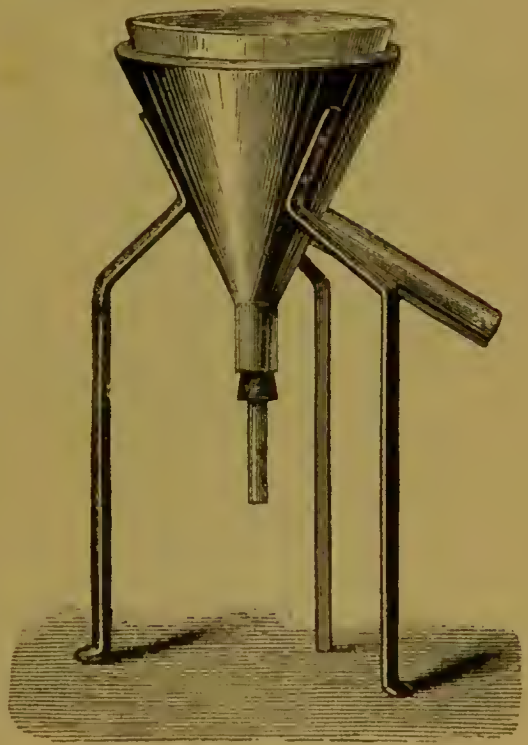

FIG. 6.—HOT-WATER FILTER

The glass filter is surrounded by a hollow copper jacket containing water heated by the flame of a Bunsen burner applied to the hollow projecting handle seen descending obliquely to the right in the figure.

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[1] The amount of gelatine may be varied with the external temperature, more being used if necessary, e.g. in very hot weather. Some habitually use 10 per cent., but, for the reason indicated, 12½ per cent. is very satisfactory for most purposes.

7. Decant into sterile test-tubes, 6 or 10 c.c. to each; plug, and sterilise on three successive days in the steamer, for ten minutes each day.

On the third day allow solidification of the gelatine to occur, half the number of tubes being kept in a vertical position, the remainder being inclined to about an angle of 10° with the horizon (see p. 9).

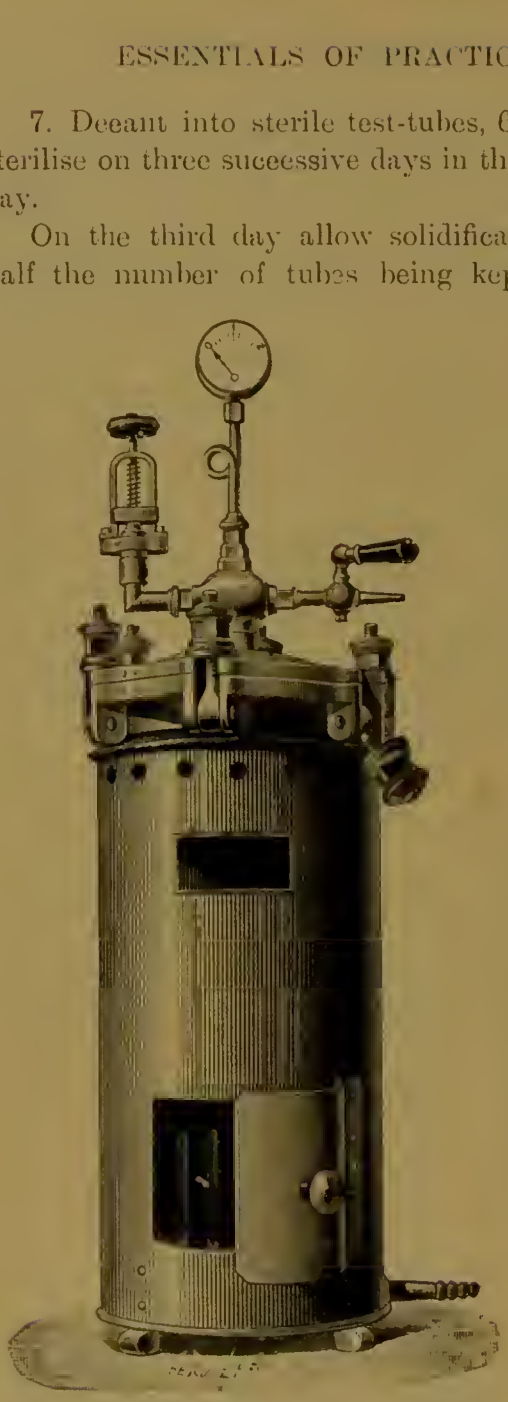

Fig. 7.—Autoclave (exterior) for the production of superheated steam

Glycerine gelatine is made by the addition of from 6 to 8 c.c. glycerine to every 100 c.c. nutrient gelatine before the final sterilisation. The addition of glycerine to the gelatine enables *B. tuberculosis* to grow on an otherwise unfavourable medium.

Sugar gelatine (Glucose gelatine) is made by the addition of 1 or 2 grammes of grape sugar (glucose) to every 100 c.c. nutrient gelatine after neutralising. Test again, and, if necessary, make slightly alkaline by adding 1 per cent. sodium carbonate solution.

This is a good medium for the cultivation of anaërobes, 1 per cent. being generally the best proportion to use.

Formate of sodium gelatine. 0·5 gramme of formate of sodium is added to every 100 c.c. nutrient gelatine before the final sterilisation.

Phenol gelatine.—The addition of $\frac{1}{10}$ c.c. of a 5 per cent. solution of carbolic acid to a tube containing 10 c.c. nutrient gelatine tends to check the growth of non-pathogenic organisms liquefying gelatine ordinarily, and does not interfere with the development of *B. typhosus*, or *B. coli*, in a mixture of organisms.

Beer-wort gelatine.—Add to 1 litre of beer-wort, preferably unhopped (to be obtained at a brewery), 100 grammes of gelatine.

Proceed as in the case of nutrient gelatine, but do not neutralise. Beer-wort gelatine and beer-wort agar (made similarly) form the best culture media for the yeasts, and are also excellent for the moulds of ringworm, favus, &c. (Hewlett).[1]

Nutrient agar-agar. 1. To the second flask, containing 300 c.c. broth set apart (p. 3) for making agar-agar, add 2 per cent. agar-agar, i.e. 6 grammes.

2. Place in the autoclave, and keep at 120° C. for three-quarters of an hour.

3. Test the reaction. If necessary, neutralise again.

4. Clarify with the white of an egg.

5. Place in autoclave again for half an hour.

6. Filter through a moistened folded filter-paper, placing the glass filter &c., with flask, into the steamer.[2]

7. The contents of the flask being now quite fluid, decant into test-tubes rapidly, about 6 c.c. into each. Plug, and sterilise for one hour in the steamer, for three successive days.

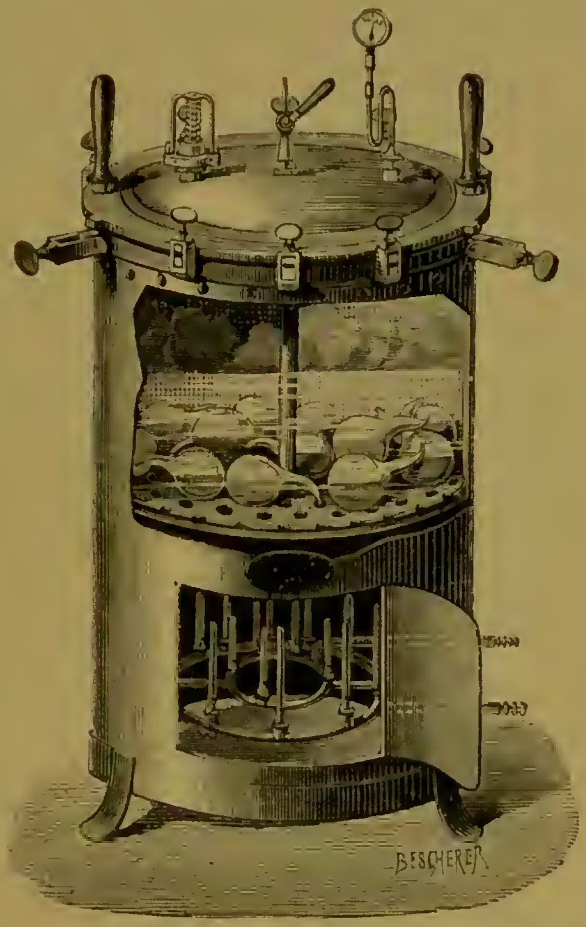

FIG. 8.—AUTOCLAVE, SHOWING INTERIOR ABOVE, AND INNER AND OUTER RINGS OF GAS-BURNERS BELOW

Both rings are used until the temperature has been raised to 120° C., and then the inner one alone is sufficient to maintain that temperature for the desired period.

On the third day place most of the tubes in an inclined position, so as to obtain a sloped or oblique surface on solidification for inoculation ('streak cultures').

The remaining tubes are to be kept upright for 'stab cultures.'

Glycerine agar is made by adding 6 grammes of glycerine to every 100 c.c. of the nutrient agar, after the medium has been kept in the autoclave three-quarters of an hour (stage 2, p. 9). The subsequent stages are the same as for ordinary agar-agar, 6 to 10 c.c. of the medium being decanted into each test-tube before sterilising, and subsequently placing them in a slanting position, in which solidification will occur rapidly.

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[1] *Manual of Bacteriology.*

[2] The hot-water filter (p. 6) may be used. But the method of filtering in the steamer is preferable in the case of agar-agar, which otherwise so rapidly cools and solidifies.

Sugar agar (Glucose agar).—After neutralising ordinary nutrient agar (stage 3, p. 9), 1 or 2 grammes glucose (grape sugar) are added to every 100 c.c. agar-agar. Boil, and note if alkaline; if not, use more sodium carbonate.

This is an exceedingly useful medium for anaërobic cultures, 1 per cent. being better than 2 per cent. glucose, generally.

Neutral litmus agar is made by adding to tubes of filtered glucose agar-agar a few drops of sterilised saturated infusion of litmus. It is most useful in demonstrating the reaction of the products of microbial activity.

Formate of sodium agar-agar is made by the addition of 0·5 gramme of formate of sodium to every 100 c.c. nutrient agar-agar before the *final* sterilisation.

Beer-wort agar-agar (see p. 9).

Potato.—1. Good-sized healthy-looking potatoes are well washed under the tap, using a scrubbing brush if necessary.

2. With a potato-borer, cylinders are bored out by steady pressure, avoiding all rotatory movement. On withdrawing the borer and pushing out the potato (by means of the left thumb), the cylinder will be found split into two symmetrical halves.

3. The ends covered with brown peel are cut off, and the potato half-cylinders allowed to soak overnight in tap water, so as to get rid of the excess of starch. If this is not done, after sterilisation the potato will be found darkened in colour, and must be rejected.

4. Each half-cylinder of potato is placed in a large test-tube, or 'boiling tube,' which has been previously fitted with a pad of wool at the bottom, plugged with another piece of wool, and sterilised.

5. Distilled water is poured in so as to cover the lower fourth of the half-cylinder. This will prevent excessive drying on sterilisation. Plug.

6. Sterilise in autoclave, at 120° C., for a quarter of an hour, once.

The alternative method of sterilising in the steam steriliser for one hour may be used, but there is a tendency to excessive drying of the medium, and a single sterilisation at 100° C. is often insufficient.

Peptone water.—1. To 1 litre of tap water, add peptone, 10 grammes; sodium chloride, 10 grammes; and shake up.

2. Boil for one hour in steamer.

3. Pour into sterile test-tubes, and sterilise on two successive days.

N.B. *Do not* neutralise.

Pasteur's solution.—The formula for this fluid, so largely used before the introduction of nutrient broth and the solid media, is as follows: Candy sugar, 10 parts; ammonium tartrate, 1 part; the ash of 1 part yeast;[1] water, 100 parts.

LESSON II (B)[2]

NUTRIENT MEDIA (*continued*)

Blood serum—Milk Elsner's potato gelatine

Blood serum.—The jugular vein of a horse is exposed with strictest aseptic precautions.

A glass cannula is inserted into the vein, and the blood allowed to flow into tall wide-mouthed cylindrical glass jars.

These are plugged with wool, or capped with some impermeable material; the jars are then set aside in a cool place.

When the clot forms and serum is squeezed out, the latter is siphoned off, preferably by the aid of an exhaust apparatus, into flasks plugged with wool. Or the wool may be conveniently replaced by a rubber stopper with a long piece of glass tubing reaching to the bottom, and a shorter one just passing through the stopper and provided with a piece of indiarubber tubing with a spring clip.

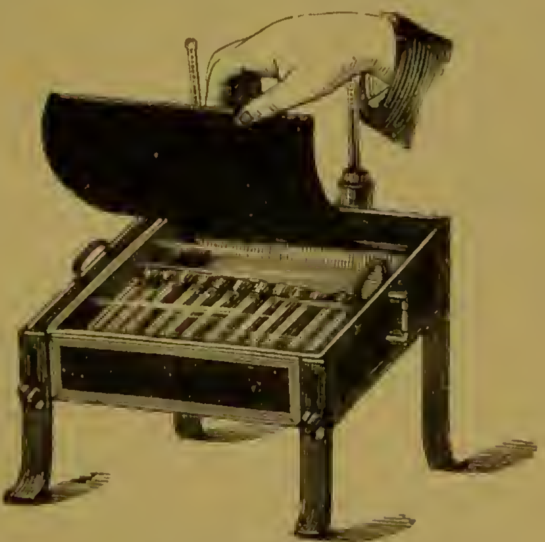

FIG. 9.—KOCH'S SERUM INSPISSATOR

The felt cover of the glass door is raised to show the tubes in position.

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[1] Bucholtz substituted for the yeast ash, potassium phosphate 0·5 gramme (Frankland).

[2] This lesson, though of practical value and therefore inserted here, may be with advantage omitted from the general course, owing to the special difficulties explained in the text, and in order to economise time.

Recently, uniformly good results have been obtained by simply aspirating the squeezed-out serum through a Chamberland or Berkefeld filter. The sterile serum thus obtained is then poured rapidly into sterile tubes, and solidified as directed on p. 12.

The flasks are then fixed in an inverted position, and the serum rapidly run into test-tubes, which are then plugged and placed in Koch's serum inspissator in an inclined position (fig. 9). They are kept at 70° C. for three-quarters of an hour, by which time the serum has firmly coagulated.

They are fitted with rubber caps, and then placed in the incubator (fig. 15) and kept at 37° C. for three days.

If no growth is visible, they are considered to be sterile, and are kept in the dark, in an ice chamber, till required for use.

N.B. Owing to the practical difficulties in carrying out the above directions, strictly aseptically from first to last, the whole batch of tubes may be found contaminated after incubation, even in the case of experienced workers. Hence, as a rule, it is far cheaper to buy the serum tubes ready for use at some good laboratory or laboratory furnisher's.

Löffler's alkaline blood serum.—This is an even more favourable medium for many organisms than ordinary blood serum, and is made by adding to every 3 parts of the serum, when in a fresh liquid condition, 1 part of a nutrient 1 per cent. sugar broth, made according to the directions on p. 4. After neutralisation, the broth is heated on a water bath, so as to separate the albuminates; it is then filtered, and sterilised in the steamer as usual. When it has cooled to 50° C. it is well mixed with the liquid blood serum and sterilised by discontinuous heating, and subsequently solidified in Koch's serum inspissator at 70° C. for three-quarters of an hour.

Serum agar-agar.—Kanthack and Stephens have recently strongly recommended the following formula for a serum agar-agar, which they find to be a good selective nutrient medium for the separation of the diphtheria bacillus from the tissues.[1] The serum agar-agar is prepared from ascitic, pleuritic, or hydrocele fluid, to which enough caustic potash is added to prevent it from coagulating on boiling. This alkaline fluid is then mixed with agar-agar (1·5 to 2 per cent.), and boiled up in the ordinary way; and finally, after filtration, 5 per cent. glycerine is added to the clear agar-agar.

Milk.—Fresh cow's milk is placed in a tall glass cylinder with a tap at the bottom, and, after standing all night in a cool place for the cream to separate, the milk is run off below the cream into sterile test-tubes. These are sterilised in the steamer at 100° C. for half an hour on three successive days. It has been recommended to facilitate the separation of cream by placing the cylinder of fresh milk at once into the steamer at 100° C. After half an hour the milk is run off into sterile test-tubes, and the sterilisation is repeated on the two succeeding days.

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[1] 'The Escape of Diphtheria Bacilli into the Blood and Tissues.' A. A. Kanthack and J. W. W. Stephens, *Trans. Path. Soc.* vol. xlvii. 1896. Details of the mode of preparation of serum agar-agar are given by these authors in the *Lancet*, 1896, vol. i. p. 835.

Other somewhat similar media are referred to in dealing with the cultivation of gonococci, p. 125 of the present work.

Elsner's potato-gelatine medium.—Used for the separation of *B. typhosus* from *B. coli communis*.

Make a decoction of 500 grammes of potato with 1 litre of water. This amount is weighed after grating the peeled potato, and it should stand in the water overnight and then be strained through butter-cloth. Boil with 15 per cent. of gelatine, i.e. 150 grammes of gelatine to the litre. Reduce the excessive acidity by the addition of 2·5 to 3 c.c. of decinormal sodium hydrate solution (i.e. a solution containing 4 grammes of the alkali to the litre of water) until the medium is of only slightly acid reaction. When cooled down to 60° C. clarify with the white of an egg. Sterilise; test the reaction; and if the acidity has increased during this operation, add more sodium hydrate until the reaction is once more only slightly acid. Filter into sterile test-tubes, 10 c.c. to each; sterilise three times as usual. Just before use, Elsner recommends the addition of 1 per cent. sterile potassium iodide, i.e. $\frac{1}{10}$ gramme to each test-tube of the medium. The advantage of adding the iodide is not obvious, and has been denied by many workers (Delépine and others).

LESSON III

COVERSLIP PREPARATIONS—METHODS OF STAINING

A. **Coverslip preparations.**—No. 1 coverslips should alone be employed, and they are best cleaned by dropping them, one by one, into a small beaker containing boiling nitric acid. After ten minutes they are taken out and washed, one by one, in tap water, and then placed in a small closed jar or petri-dish, 1½ inch in diameter, containing alcohol.

The coverslip is so thin that the least force applied to the edge of the glass, so as to bend it ever so slightly, may be sufficient to break it.

The following method is elaborate, but its careful practice will save many coverslips and much annoyance.

1. **To clean the coverslip.**—(*a*) A clean rag[1] or silk handkerchief, freed from grease, is placed over the palmar surface of the left hand, and a fold pushed well into the angle between the thumb and index finger.

(*b*) With fine forceps a coverslip is taken from the jar of alcohol and placed on the rag, and it is then held between the left thumb and middle finger (both covered of course with the rag) and supported laterally by the index and ring fingers.

(*c*) By a circular movement of the thumb, the apposed surface of the coverslip is cleaned. The latter is now reversed, so that the opposite surface of the glass can be cleaned by the movement of the thumb.

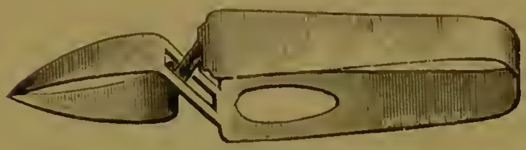

FIG. 10.—CORNET SPRING-CLIP FORCEPS

(*d*) The central part of both surfaces of the coverslip is now clean, and the peripheral portion can be dealt with by rotating the coverslip between the left thumb and middle finger, by means of the same digits of the right hand applied to the edge.

(*e*) Finally, any fluff from the rag must be blown away. The coverslip is held horizontally by means of a pair of cornet forceps, the lower limb of which is perforated by an opening not unlike a keyhole in shape. We may therefore briefly indicate this as the 'keyhole' side of the forceps (fig. 10).

2. **To make a film preparation.**—(*a*) With a platinum loop (fig. 11) sterilised by heating to redness in the Bunsen flame, a drop of distilled water, kept at hand in a watch-glass, is transferred to that surface of the cleaned coverslip which corresponds with the 'keyhole' side of the cornet forceps.[2]

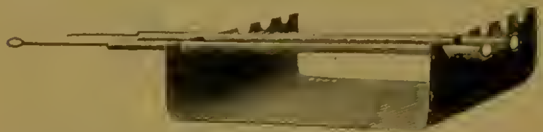

FIG. 11.—TIN RACK WITH GLASS-HANDLED PLATINUM STAB-NEEDLE, AND LOOP (WHICH HAS BEEN DRAWN TOO LARGE, PROPORTIONALLY)

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[1] Butter-cloth (see footnote, p. 4) is recommended, as it is comparatively free from fluff, and, being inexpensive, may be thrown away when too dirty for further use.

[2] The film surface should, *as a routine practice*, always correspond with the 'keyhole' side of the cornet forceps; and in taking up a prepared coverslip, laid down at any stage, the same plan should be adopted, the coverslip being picked up so that the film and 'keyhole' correspond in direction. The advantage of thus always knowing 'which side the film is on' will soon be appreciated, especially in the case of Van Ermenghem's method for staining flagella. In this method silver nitrate is used, so that a mirror-like surface is presented on both sides of the coverslip, in place of the lustreless appearance usually distinguishing the film side from the other.

(*b*) A little of the culture given round (e.g. *Staphylococcus pyogenes aureus*) is now to be transferred from the culture tube to the drop of water on the coverslip. To avoid contaminating the culture by allowing air organisms to fall in, or be conveyed in by means of the platinum loop, the following 'ritual,' as it has been called, must be observed.

The culture tube being held between the left thumb and index finger, with the lower end directed away from the operator, so that the upper inch or so rests against the thenar eminence and the sloped surface of the medium is clearly seen, the wool plug is quickly burnt in the Bunsen flame; then with dissecting forceps (the points of which have also been passed through the flame) the plug is by a rotatory movement withdrawn for about a quarter of an inch, and burnt a second time. By means of a similar rotatory movement the plug is withdrawn and placed between the index and middle fingers, the tube being kept in a more or less horizontal position if it be solid, and slightly inclined from the vertical if it be liquid.

The glass-handled platinum loop, held in the right hand like a pen, is passed through the Bunsen flame, the loop itself being downwards. By a rapid movement obliquely upwards and downwards through the flame, both loop and glass handle, except where held, are sterilised, the wire itself becoming red-hot.

The loop must be allowed to cool, otherwise the culture may be killed. If too much time is allowed for this purpose, however, air organisms may be carried in on the cold loop.

The plug being withdrawn, the sterile platinum loop is inserted into the culture tube and a *minute quantity* [1] of the growth removed. The plug is sterilised by passing it through the flame, and then re-inserted into the tube. The prepared coverslip, with a drop of sterile water on its under surface, is then smeared with the growth on the platinum loop, and the watery emulsion thus produced uniformly distributed over the coverslip. This is allowed to dry in the air, or by warming the coverslip at some distance above the flame. Such a film is dried, but not permanently fixed to the coverslip. There would be great risk of washing away the film if it were placed in water at this stage.

To fix the film, the coverslip, held in cornet forceps, is passed horizontally and rapidly through the flame three times. This coagulates the minute quantity of albumen present, and the film is thereby permanently fixed. Subsequent staining and washing may be safely undertaken without any fear of the film being washed off the cover-slip.

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[1] Just about as much as would be removed by the *point* of a platinum stab-needle, which, indeed, may be used instead of the loop.

B. **Stains and staining.**—The methods of staining may be divided into two main classes:

1. Simple.
2. Compound, or more or less complicated,[1] including
   (*a*) Gram's method.
   (*b*) Ziehl-Neelsen's method for tubercle bacilli, &c.
   (*c*, *d*, &c.) Spore and flagella staining, and other more special methods.

1. *Simple staining.*—By this is meant pouring on to the film a stain, and after a certain number of minutes pouring it off and washing carefully and thoroughly in water. The time must be sufficient to stain the organism distinctly; the subsequent washing must be thorough enough to wash out all superfluous stain, so that in a film made with a pure culture only the bacilli are stained, the space intervening between individual bacilli being colourless. This is what is meant by making 'a clean preparation.'

To stain distinctly and to obtain a clean preparation afterwards, will require varying periods of exposure to the dye and subsequent washing. In other words, the staining and washing times vary with the intensity of the stain employed.

Stains.—The following anilin dyes are in most general use, and are quite sufficient for ordinary purposes. Many others, of course, might be included in the list; and of these, *thionin* (p. 256) is, perhaps, the most deserving of special mention.

1. Löffler's alkaline methylene blue ('Löffler's blue').
2. Carbol-methylene blue ('Kühne's blue').
3. Anilin-water gentian-violet ('gentian-violet').
4. Watery fuchsin ('fuchsin').
5. Carbol-fuchsin.
6. Anilin-water fuchsin.

Formulæ of stains.—Saturated alcoholic solutions of crystals of methylene blue, fuchsin, and gentian-violet are kept in stock.

1. *Löffler's methylene blue* is made by adding to 100 c.c. of a solution of potash (1 in 10,000) 30 c.c. saturated alcoholic methylene blue.

2. *Kühne's blue*, or *carbol-methylene blue*, is made[2] according to the

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[1] These 'compound' methods, as well as those for section-staining, are described subsequently as the need for them arises. But after facility in simple staining has been acquired, Gram's method (see pp. 96, 97) may be conveniently practised at this stage if suitable organisms, e.g. a *Staphylococcus*, be given round the class.

[2] More accurately, 1·5 grammes of methylene-blue crystals are dissolved in 10 c.c. absolute alcohol, and this solution added to 100 c.c. of a 5 per cent. solution of carbolic acid. So for carbol-fuchsin solution, 1 grm. of the crystals of fuchsin being substituted for 1·5 grm. methylene blue.

formula given below for *carbol-fuchsin*, a concentrated alcoholic solution of methylene blue being used instead of fuchsin. This is a rather more intense stain than Löffler's blue.

3. *Anilin gentian-violet.*—First prepare 'anilin-water' by shaking up into an emulsion a mixture of 5 c.c. anilin oil and 100 c.c. distilled water.

Filter through *moistened* filter paper. The filtrate should be quite clear and free from oil globules. *Dry* filter paper would not keep the latter back.

To 100 c.c. anilin-water add 10 c.c. absolute alcohol, and 11 c.c. concentrated alcoholic gentian-violet. Keep in a stoppered bottle.

4. *Watery fuchsin.*—Concentrated alcoholic solution of fuchsin crystals is added to a bottle three-quarters full of distilled water, until, after shaking the bottle, the solution is no longer translucent.

5. *Carbol-fuchsin.*—To 100 c.c. of 5 per cent. carbolic acid add concentrated alcoholic solution of fuchsin until a metallic lustre appears on the surface, and the solution has lost its translucency.[1]

6. *Anilin-water fuchsin* is made according to the formula given above for anilin gentian-violet, a concentrated alcoholic solution of fuchsin being used instead of gentian-violet.

Of these six dyes, perhaps the most frequently used are Löffler's methylene blue, anilin-water gentian-violet, and carbol-fuchsin. Each stain should be kept in a wide-mouthed bottle, fitted with a small filter and filter paper, passing through the rubber stopper (fig. 12). Anilin gentian-violet so readily decomposes in contact with the air, that it is a good plan to keep it in a stoppered bottle, having *another* bottle, fitted with filter, &c., kept in the staining stand, so that the filter is ready for immediate use.

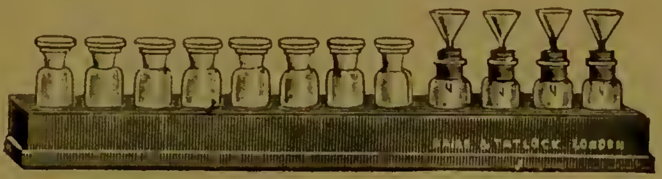

FIG. 12.—STAND FOR STAINING REAGENTS

Four of the bottles are fitted with glass filters passing through rubber stoppers.

N.B. It is absolutely essential to filter all anilin dyes, and the stain should be enough to completely conceal the coverslip; otherwise an unsightly ring of deposit may remain obvious on the glass, and be difficult to wash away.

The times required for staining.—These naturally vary inversely with the intensity of the stain employed.

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[1] The turbidity, which is sometimes noticed on mixing these solutions, can be obviated by warming the stain for half an hour in the steamer.

As a general rule, the following may be taken as the average times for staining film preparations : Löffler's methylene blue, 5 or even 10 to 15 minutes ; anilin gentian-violet, 2 to 2½ minutes ; carbol-fuchsin 1½ to 2 minutes.

Very little subsequent washing in water is necessary in order to obtain a clean coverslip preparation after staining with Löffler's blue, whereas with carbol-fuchsin, as also (but to a less extent) with gentian-violet, a very thorough washing under the tap is necessary ; or, with these intense stains, the coverslip in forceps may be allowed to soak in a bowl of water for ten or fifteen minutes, depending upon the thickness of the film and the time it has been exposed to the stain.

Drying and mounting the coverslip preparation.—During the making, staining, and washing of the film preparation, the coverslip has been held in the cornet forceps, with the film surface upwards, corresponding with the 'keyhole' side of the forceps (see footnote, p. 14). Pieces of 'German blotting-paper,' conveniently cut to the size 4″ × 5″, are kept at hand, and one of these is folded in half, and this again into half ; so that there is a pad of four thicknesses, i.e. two folds each of double thickness.

After draining off the excess of water by holding the coverslip in forceps edgewise on the pad, the paper is opened out and the coverslip is placed, film side up, on the lower fold of double thickness—i.e. that next to the table—and the other double fold is pressed gently down on to the coverslip. This absorbs most of the moisture. *After drying the points of the forceps*, the coverslip is then removed to a fresh piece of the same pad and completely dried, by again applying gentle pressure upon the upper fold of the pad. The coverslip is taken up in the cornet forceps, the film surface, of course, corresponding with the keyhole side of the forceps, and placed carefully down upon a small drop of a solution of Canada balsam in xylol in the centre of a clean slide.

After a few moments, excess of balsam, and any air-bubbles present, are removed by carefully gripping slide and coverslip together with cornet forceps.

The slide should be *at once* labelled with the name of the preparation, method of staining, and the date.

LESSON IV

METHODS OF CULTIVATION

Liquid media.—Nutrient broth, peptone water, milk.

Hold the tube to be inoculated, together with that from which the culture is to be made, between the thumb and index finger of the left hand, so that the surface of the agar-agar growth (preferably) from which the cultivation is to be made is clearly visible.

The 'ritual,' as it has been called, referred to in Lesson III. (p. 15), of burning the wool plugs twice before removing the latter, has to be observed every time a fresh cultivation is made. Care is to be taken in the case of liquid media, during this passage of the plugs through the flame &c., that the fluid is not allowed to reach and moisten the wool. This is avoided by holding the tubes only slightly inclined from the vertical during the process of burning the plugs.

After this has been done, and the plugs of the two tubes have been withdrawn and held between the second and third, and the third and fourth fingers, respectively, the platinum loop is sterilised in the way referred to (p. 15); and when sufficient time has been allowed for it to cool, a small quantity of the culture is removed and inserted into the tube containing the liquid medium. The best way to inoculate *fluid* is to begin rubbing the loopful of culture on the glass itself, immediately above the line of the liquid, inclining the tube gradually, until the material first rubbed into the glass becomes submerged, and at the same time passing the loop a little farther into the fluid. The reason for not placing the loopful of growth directly into a liquid medium is that the culture often adheres so tightly to the loop that it is difficult to detach it. Rubbing it first into the glass and then gradually into the liquid obviates this difficulty.

When the inoculation has been made, the platinum loop is withdrawn, the plugs burnt in the flame and replaced in their respective tubes, and—*without having laid it down in the interval*—the loop needle is thoroughly sterilised in the flame, and placed in the rack provided (fig. 11). The tube inoculated is at once labelled with the name of the culture, and the date.

Solid media.—Nutrient gelatine, agar-agar, potato, potato gelatine, serum, &c.

These media can in most cases be inoculated in two ways, according to the position in which they have been allowed to set or harden. They may be solid cylinders, the so-called 'stab gelatine,' 'stab agar,' &c., in which case a straight needle inoculated with a culture is passed directly down the centre or core of the cylinder, producing, in time, the 'stab cultivation.'

But if the medium has been allowed to solidify so as to present an oblique surface (gelatine, agar, serum, &c.; or, in the case of potato, split so as to present a flat surface), this is inoculated by spreading a little of the growth over it, and it is then known as a 'streak cultivation,' and the medium is termed an 'oblique' or 'streak gelatine,' &c.

Method of inoculating a stab gelatine, &c.—If the growth from which the inoculation is to be made is on a solid medium, both it and the stab gelatine to be inoculated are held between the thumb and index finger of the left hand horizontally, with the surface of the tube containing the growth to be inoculated, rotated so as to be clearly visible. With the usual precautions as to burning the plugs twice, removing them, placing them between the fingers and sterilising the stab-platinum needle, a small portion of the culture is removed, and the needle then carefully passed along the centre or core of the cylinder of gelatine, nearly if not quite to the bottom of the tube. It is steadily withdrawn, care being taken, especially as the point emerges from the gelatine, not to split the medium[1] by any jerky movement of the needle. Plug and label the tube as usual, not forgetting to sterilise the needle.

Method of inoculating a streak or oblique gelatine, &c.—The usual precautions being taken as to burning the plugs twice and sterilising the needle, a small portion of the growth is removed on the platinum loop, which is inserted gently, and as far down as possible, parallel with, and close to, the surface of the gelatine: as it is withdrawn, the growth on the loop is spread over the surface of the gelatine in a straight line.

Care is to be taken to have a perfectly flat loop to start with, and to avoid digging into the medium itself by tilting one or other edge of the loop out of a plane parallel with that of the surface to be inoculated. Plug and label the tube, &c., as usual.

OTHER METHODS OF CULTIVATION[2]

Shake cultivation—Esmarch tube-culture or 'roll-tube'—
Plates and petri-dish preparations

'Shake' cultivation.—This is the method used for demonstrating the formation of gas, and is especially useful in distinguishing between the common organism of the intestine (*Bacillus coli communis*) and the bacillus of typhoid fever (*Bacillus typhosus*)—the *Bacillus coli* produces gas, the typhoid organism being characterised by its inability to do so. (See pp. 173, 233).

Method of making a 'shake' cultivation.—Melt a tube of nutrient gelatine in a water bath (see fig. 18, p. 24), and when the temperature, as indicated by the thermometer, is at, or just below, 37° C., inoculate it (as if it were a tube of broth) with a loopful of growth from a tube containing *B. coli communis*.

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[1] Should the medium split on entering the needle, it is a sign that the gelatine has been too long in the tube. It should be remelted and allowed to solidify again, when this phenomenon will no longer be noticed, unless the medium has become very dry from old age.

[2] Special methods for cultivating **anaërobes** are dealt with in Lesson XX., p. 222.

Holding the tube vertically between the palms of the two hands, rotate steadily; avoid making bubbles by any vigorous shaking up. Rapidly solidify by placing under a stream of water from the tap, or in a narrow jar of cold water. After capping the tube, incubate at 20° C.

Agar-agar, and (better still) sugar-agar, may also be used in making shake cultures of *B. coli*, or, in the case of the anaërobes, the bacillus of malignant œdema, and of quarter-evil, &c. The temperature, after thoroughly liquefying the agar (preferably in the autoclave at 120° C.), must be cooled down to about 42° C. in a large water bath full of water. The inoculation with the growth, and subsequent rotation of the tube whilst in the vertical position, must be more rapidly done than when gelatine is used, owing to the rate at which agar-agar solidifies below about 40° C. When capped, the tube is kept at 37° C.

Esmarch's roll-tube.—This was formerly much used for separating mixed cultures or counting the colonies in a sample of water. Its use has been largely given up in favour of the far more convenient petri-dish preparation.

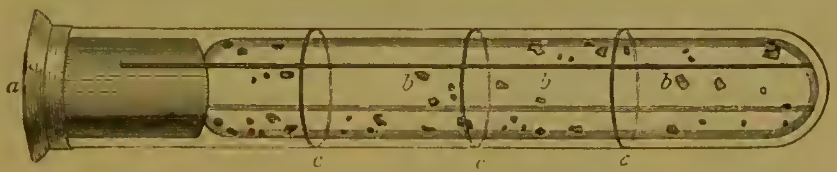

FIG. 13.—ESMARCH TUBE-CULTURE OR 'ROLL-TUBE'

a, india-rubber cap; *b*, *b*, *b*, longitudinal line drawn on glass; *c*, *c*, *c*, transverse lines on glass to facilitate the counting of the colonies. [Frankland.]

To make an Esmarch's roll-tube.—Gelatine is the easier to manipulate, though, with the precautions already mentioned, agar may be equally well employed for making roll-tubes.

It is essential that only a small quantity of gelatine should be used—about enough to occupy the bottom half-inch of the $5 \times \frac{5}{8}$ inch tube (about $1\frac{1}{2}$ c.c.). The gelatine is either first inoculated by stabbing the solid medium, and then melted at or below 37° C., or melted and then inoculated at, or below, the body temperature.

The tube is capped, but before attempting to solidify the gelatine, and in order to form a thin uniform layer throughout the interior of the tube, the liquefied medium is allowed to run over and moisten completely the inner surface, until every little islet of glass has been exposed to the gelatine. If this is not carefully done, on placing the tube in cold water and rolling, there may be areas left on the inner surface of the tube uncoated with gelatine.

Solidification is accomplished by holding the capped tube nearly horizontally (not quite, to avoid contact of the gelatine with the wool plug) in a large basin of water. Ordinary tap water may be used, and this insures a more uniform distribution of the medium, perhaps, than if ice is added; though the addition of ice renders the operation more rapid and less tedious. The objection to using a block of ice for this purpose, as recommended by some, is that there is a great tendency for the gelatine to solidify at the first place where the tube comes in contact with the ice; so that a uniform lining with gelatine—i.e. an even distribution of the colonies—becomes difficult, if not impossible.

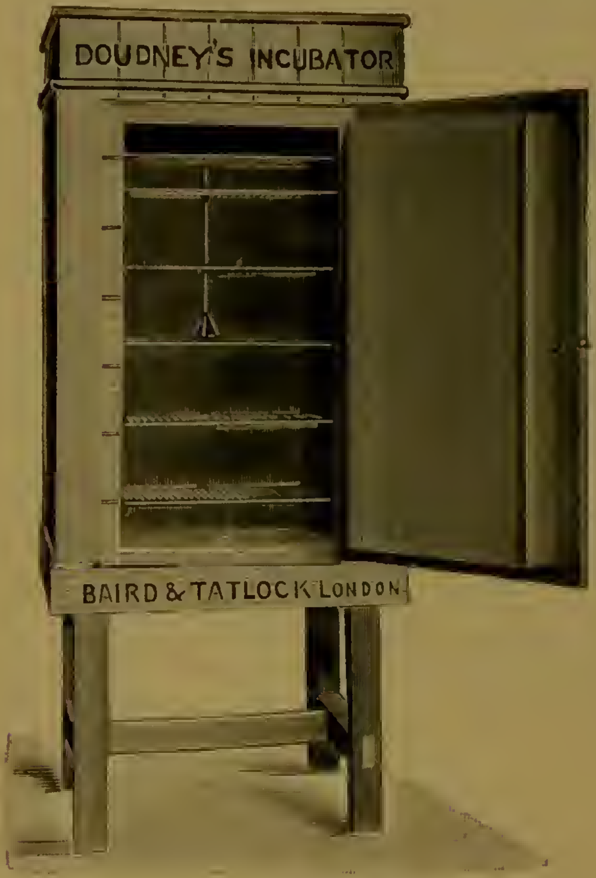

FIG. 14.—DOUDNEY'S INCUBATOR FOR LOW TEMPERATURES (20° to 22° C.)

There is a tank of water (not shown) below the lowest shelf, warmed by a flame beneath the cupboard; and resting on the tank is a coil of tubing through which cold water circulates. By means of a regulator, a uniform temperature is maintained.

When solidification is complete, the tube is kept at 20° C., if gelatine is used; or at 37° C. in the case of agar-agar.

It may be mentioned here, that an incubator, or 'gelatine cupboard' (fig. 14), for low temperature (20° to 22° C.), has recently been introduced by Mr. Doudney, of the Royal College of Surgeons and Physicians' Laboratory, London, which is simple and most efficient in maintaining a uniformly low temperature. All other media but nutrient gelatine are to be kept at 37° C., and Hearson's incubator (fig. 15) is strongly recommended.

Petri-dish preparations; plate preparations.—Formerly, sterile oblong glass plates were covered with previously inoculated medium, gelatine or agar-agar, and kept in a sterile glass dish covered by a bell-jar at the appropriate temperature. The use of these plates has now been almost completely given up in practical work, petri-dishes (fig. 17) being far more convenient than the former. But though no longer much in use, the name survives, and 'plates,' or 'plate preparations,' is the usual laboratory term for the petri-dish preparations—and we shall adopt this well-known meaning throughout this book.

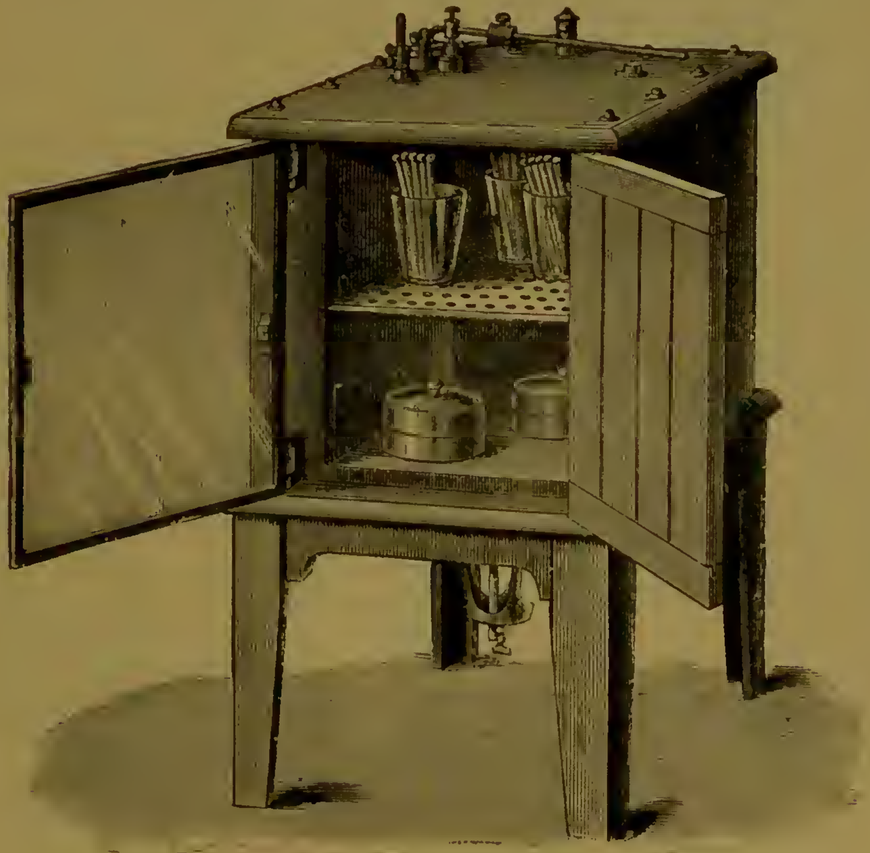

FIG. 15.—HEARSON'S INCUBATOR FOR WARM TEMPERATURES (37° C.)

Method of making plate (i.e. **petri-dish**) **preparations.**—Gelatine or agar-agar, or their modifications—potato gelatine &c.—may be employed. Sterilised petri-dishes packed in sterile paper are kept ready for use.

Gelatine plates.—A water bath with copper test-tube rack and thermometer are made ready (fig. 18). Three tubes of nutrient gelatine are melted down, and when the temperature has been lowered, if necessary, by the addition of cold water, to below 37° C., one of the tubes is inoculated with a loopful of culture, &c.

The plug being inserted, and the needle sterilised in the usual way, the tube is rotated between the palms of the hands, keeping the former in a vertical position the whole time, and avoiding the production of bubbles.

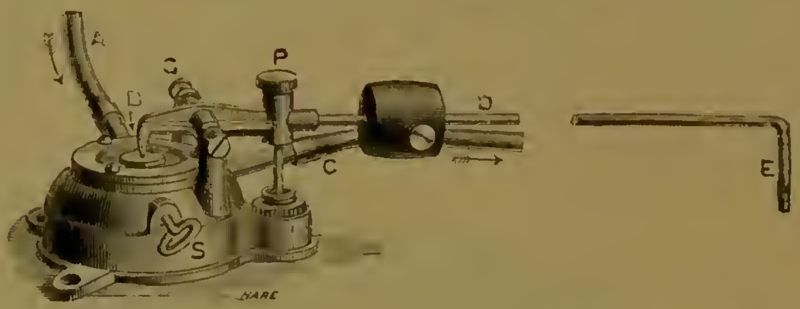

Fig. 16.—HEARSON'S PATENT REGULATOR FOR MAINTAINING A UNIFORM TEMPERATURE IN THE INCUBATOR SHOWN ON P. 23

The tube is marked 'Original,' with a glass pencil,[1] and replaced in the warm bath for about half a minute, to insure it being thoroughly liquefied.

It, together with another liquefied gelatine tube, is taken out of the bath and *one* loopful of the contents of the 'Original' tube transferred to the second tube, which is marked 'No. 1,' rotated, and returned with the Original to the bath.

The third tube is then inoculated, using now *three* loopfuls of the contents of tube No. 1, which is greatly diluted compared with the Original. This third tube is marked 'No. 2,' and rotated &c. as in the case of the other two tubes.

Fig. 17.—PETRI CULTURE-DISH

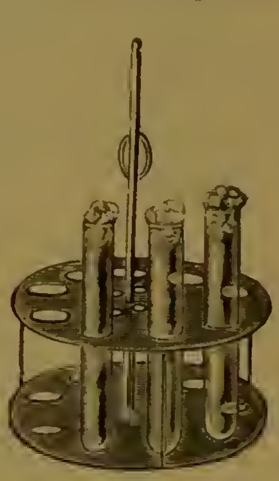

Fig. 18.—COPPER TEST-TUBE RACK, WITH THERMOMETER AND TEST-TUBES, FOR USE WITH A WATER BATH

The petri-dishes are now removed from their paper covers.

The Original tube is taken from the bath, the plug burnt twice, as in the case of inoculating a tube (p. 15), and the neck allowed to cool.

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[1] A 'glass pencil' is a coloured pencil in which the pigment is mixed with a soft wax, so as to adapt it for writing on glass. It is a great convenience, and may be obtained from Messrs. Baird & Tatlock, 14 Cross Street, Hatton Garden, E.C.

Holding the tube in the right hand, the plug is seized between the little finger and hypothenar eminence of the left hand and twisted right out. Immediately after, the top of the petri-dish is grasped between the left thumb and index and middle fingers and rapidly lifted up, so as just to permit the neck of the tube to be inserted between the cover and the petri-dish proper. The gelatine is poured out quickly, the cover replaced, and the medium allowed to spread evenly over the surface of the dish. With a glass pencil, the cover of the dish is marked with the name of the cultivation, the number of the tube from which it was plated, the date, and the initials of the worker—e.g. '*B. typhosus*, Original (or 1st dilution, &c.), July 20, 1900, A. B. C.' Then No. 1 tube, known as the 'first dilution' tube, is plated similarly; and so for No. 2, the 'second dilution.'

All three petri-dishes, or 'plates' as they are commonly called, are kept on a level surface in a cool place, e.g. a cold stone slab, until they have thoroughly solidified, and then they are packed up in the sterile paper from which they were removed at the commencement, and placed in the gelatine cupboard at 20° C. Since dew may collect in considerable quantity during the first twenty-four hours, and in this case may run down from the cover to the surface of the gelatine, spoiling the appearances of the colonies, it is often advisable, during the first twenty-four hours' incubation at any rate, to keep the dishes with the cover side *down*. Subsequently, especially in the case of an organism liquefying gelatine, the dish may be kept with its top uppermost.

Agar-agar plates are made on the same general plan as in the case of gelatine, but allowance must be made for the fact that agar does not thoroughly melt much under 100° C. The following precautions should therefore be taken, otherwise plating with this medium will be difficult, if not impossible, the agar solidifying at about 38° C., so that it can no longer be poured out from the tube.

Precautions to be adopted.—Fill the water bath full of water and keep it at 42° C. Use tubes containing plenty of agar-agar—half full, in the case of a tube 5 inches long by $\frac{5}{8}$ inch in diameter, for a petri-dish 3 inches in diameter.

Thoroughly melt three such tubes down over the flame, or preferably in the autoclave at 120° C. When about to use them, transfer to the large water bath, already prepared.

When the temperature is about 42° C., rapidly inoculate and rotate the first or 'Original' tube from the given material (culture, &c.).

Transfer one loopful from the 'Original' tube to 'No. 1,' and three loopfuls from 'No. 1' to 'No. 2' tube, just as in the case of gelatine plates.

Solidification occurs rapidly. The cultures are then incubated at 37° C.

It should be mentioned that **another way of inoculating an agar plate** is to pour the thoroughly liquefied agar-agar into the petri-dish with the usual precautions. When the agar is quite solid, the cover is lifted just high enough to allow a platinum loop inoculated with the given material to be inserted. A series of parallel streaks are then made, beginning at the side of the dish farthest away, and ending up at the near side. The last streak made will contain only a few discrete colonies.

A previously sterilised **camel's-hair brush** may sometimes be conveniently substituted for the platinum loop, the surface of the medium being gently brushed in parallel streaks (see p. 182).

LESSON V

METHODS OF EXAMINING THE CULTURES AND ENUMERATING THE COLONIES—THE EXAMINATION OF COVERSLIP PREPARATIONS—THE HANGING DROP—IMPRESSION PREPARATIONS—HOW TO STERILISE A CULTURE

The examination of cultures.—This has first to be done with the unaided eye, noting the appearance presented by the colonies—colour, outline, moisture or otherwise of the surface, &c.

A hand lens is useful in making out the exact condition of the edge or periphery of a colony.

The low power ($\frac{2}{3}$-inch objective) may often be advantageously used even for examining an oblique tube cultivation, in its upper part especially, where the medium is thinnest.

Esmarch's roll-tubes are examined in the same way; but a special apparatus, consisting of a lens in a holder which can be fitted on to the tube, was originally employed by Esmarch for counting and examining the colonies.

This is not indispensable, however, and a simple way to count the colonies resulting from inoculating a known quantity of, say, water is to divide the tube longitudinally into four equal areas by means of a glass pencil. Then lines are drawn at right angles to the longitudinal ones, i.e. horizontally, dividing the whole surface of the glass tube into a number of quadrilateral areas as nearly equal in size as possible (fig. 13).

The number of colonies in ten such areas is noted, and the average for an area obtained. The superficial extent of the glass tube is readily estimated, and so the number of colonies which have developed from the known quantity of liquid can be enumerated.

Plates.—The colonies are examined through the bottom of the inverted petri-dish, the medium being generally translucent enough for this purpose. If liquefying organisms are present, careful manipulation is needed—rapidly turning the dish bottom upwards—to avoid spreading about gelatine already liquefied, and loss of the characteristic appearance.

The dish, with its cover side down, is then to be placed on the stage of a microscope and examined with the $\frac{2}{3}$-inch objective, and characteristic colonies drawn, for practice.

To enumerate the colonies on a 'plate' (petri-dish) cultivation **Wolffhügel's apparatus** has, hitherto, been generally used. This consists of a wooden stand on which rests a piece of black glass. A square piece of glass, ruled into squares—each side of which is 1 centimetre in length—rests upon supports immediately over the black glass plate. Some of the squares are still further divided up into smaller squares, each side of these being $\frac{1}{10}$ centimetre in length.

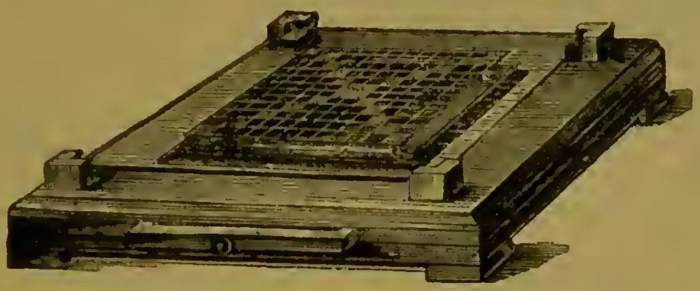

FIG. 19.—WOLFFHÜGEL'S APPARATUS FOR COUNTING COLONIES IN A PLATE CULTIVATION

Method of using.—Remove the ruled piece of glass; place the petri-dish, cover side down, on the sheet of black glass; replace the ruled piece of glass so as to cover the dish.

The colonies show up well against the black background. If not too numerous, the colonies in the larger squares are alone counted. Ten squares are thus counted, and an average struck. The area of the dish can easily be estimated by means of the graduated glass, and so the number of colonies which have developed as the result of inoculating a known quantity of material can be enumerated. If the colonies are more numerous, the smaller squares are made use of, and a hand lens employed to count the minute growths visible. When the colonies are too thickly scattered for ready enumeration, it will be necessary to use less of the original material to be investigated, as will be explained later.

Pakes's enumerating disc.[1]—This simply consists of a circular piece of blackened cardboard, or paper (fig. 20), divided into sixteen equal sectors. Each of the sectors, numbered 1 to 16, is $\frac{1}{16}$th of the whole disc; the colonies overlying complete sectors should therefore be counted, and the number thus obtained divided by the number of sectors counted, and multiplied by 16. The two smaller circles and the subdivisions in sectors 1 and 2 are merely for ease in counting. This method is rapidly replacing the use of Wolffhügel's apparatus by reason of its greater accuracy, ease, and very low price.

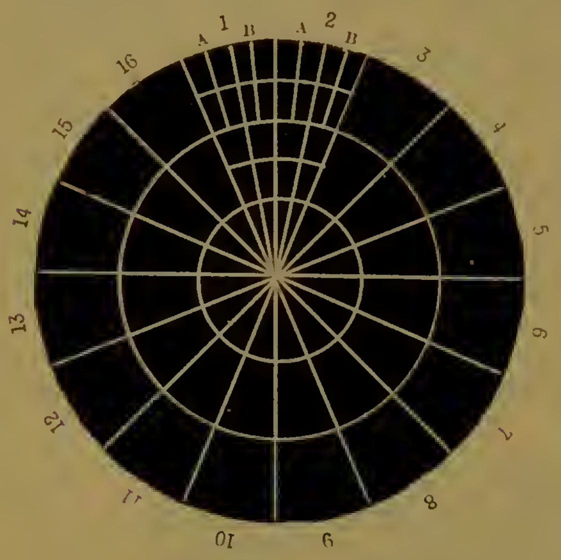

FIG. 20.—PAKES'S ENUMERATING DISC

To make a pure culture from a mixed plate.—The cover of the petri-dish is removed, and the colonies examined by means of the $\frac{2}{3}$-inch objective, the free end of which is now inside the dish but does not touch the medium. The colony, which is to be removed so as to make a pure culture, being focussed, the point of a sterilised platinum stab needle is first placed close to the growth, so that it is now visible in the field of the microscope on examination. With the greatest care, while still observing with the microscope, an attempt is made to dip the point of the needle right into the colony; if successful, the needle is quickly transferred to a tube of culture medium, and a pure culture thus obtained. This method requires some practice, but is the one generally in use, e.g., for the separation of the *Vibrio choleræ asiaticæ* from contaminated water.

Another way is to focus the suspected colony, and when it is in the centre of the field of the microscope, the $\frac{2}{3}$-inch objective is removed and replaced by a similar brass extremity, into which a fine 'harpoon' of metal is adjusted so that its point corresponds accurately to the centre of the objective mentioned. By means of the coarse adjustment, the harpoon point is made to dip into the colony, and a subculture made by streaking it over the appropriate medium.

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[1] May be obtained from Messrs. Baird & Tatlock, 14 Cross Street, Hatton Garden.

Examination of coverslip preparations.[1]—*The use of the $\frac{1}{12}$-inch oil-immersion lens.*—It is presumed that the student is familiar with the use of the lower powers ($\frac{2}{3}$-inch, $\frac{1}{6}$-inch) of the microscope. When examining a coverslip preparation for micro-organisms, first lower the Abbé condenser, by means of the screw attached, to a distance of about a third of an inch beneath the stage, and, using the $\frac{2}{3}$-inch objective, see if the film is sufficiently stained and sharply defined. When a favourable-looking region is found, the low power is rotated away by means of the nosepiece, so that the $\frac{1}{12}$-inch oil immersion takes its place ; the condenser should then be screwed up as high as possible. A small quantity of cedar oil is dropped, by means of a pointed glass or metal rod, on to the coverslip just where this will be touched by the immersion lens. The coarse adjustment is then used to lower the objective until it meets the surface of the oil, which at once runs up over the lens, owing to capillary attraction. This rapid movement of the oil serves, in fact, to indicate the moment of contact between the lens and the surface of the oil. Henceforth only the fine adjustment is to be used in focussing the preparation.

The advantage of the oil-immersion lens.—Owing to the difference between the indices of refraction of glass and air, some of the rays of light reflected up through the condenser and coverslip preparation tend to spread outwards, i.e. away from the object glass, the more central rays alone passing through the lens on their way to the eye of the observer. There is thus a loss of illumination, which is greater as the magnifying power of the objective is increased, so that it is more noticeable with a $\frac{1}{6}$-inch than with a $\frac{2}{3}$-inch, and still more with a $\frac{1}{12}$-inch lens. One obvious reason is that the small size of the lowest lens composing the composite $\frac{1}{12}$-inch objective, compared with that of lower magnifying powers, prevents all but the more centrally directed rays passing through it.

The index of refraction of cedar-wood oil so nearly approaches that of glass, that loss of illumination, arising in the way mentioned, is greatly minimised by its use.[2]

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[1] For a short account of the microscope, see Appendix A, p. 271.

[2] The density of the cedar-wood oil is increased by allowing it to evaporate until its index of refraction has approached that of glass as nearly as possible.

As a substitute for cedar-wood oil, in an emergency, glycerine and water, equal parts, may be employed, or even water alone, though the loss of illumination is in this case considerable.

The hanging drop is a preparation in which a drop of a fluid culture[1] is placed on the under surface of a clean coverslip, and allowed to hang down into the hollow of a hollow-ground slide. By its means the motility of organisms can be well demonstrated. By a slight modification—transferring a drop of liquid nutrient gelatine or agar-agar to the under surface of a coverslip, inoculating it with a given organism, and fixing it over the hollow of the slide—the progress of the growth, together with the formation of spores, and the development of the spores again into bacilli, can be watched at regular intervals.

Method of making a hanging drop.—The circumference, to a distance of about $\frac{3}{16}$ inch, of the hollow in a clean hollow-ground slide is ringed with vaseline, which is applied by means of a camel's-hair brush.

The cleaned coverslip is fixed in cornet spring forceps. With sterile platinum loop some broth[1] culture is transferred to the centre of the under surface of the coverslip, and the latter quickly placed over the hollow of the hollow-ground slide, taking care that none of the broth touches the vaseline at the edges, which serves to fix the coverslip fairly well, and prevents evaporation.

Method of examining a hanging drop.—The hanging-drop preparation differs in several respects from an ordinary coverslip preparation, and special care has to be taken in its examination to avoid breaking the coverslip and so getting the broth culture, which may be virulent, over the immersion lens, &c. The following points are to be noted:

1. In a hanging drop the coverslip is only supported at its circumference, and the larger more central portion, being unsupported, yields, when the oil-immersion lens is screwed down on to it, up to a certain point, and then suddenly snaps.

Some kinds of cedar-wood oil sold by continental makers are, however, apparently thickened by the addition of Canada balsam, which becomes deposited on the coverslip, the objective, &c., greatly to the detriment of the lens. Good 'watchmaker's oil' is perhaps the best fluid to use.

After the slide has been examined the oil may be at once wiped off with a rag, care being taken to avoid shifting the position of the coverslip before the Canada balsam has hardened. If the preparation is left several days, the dried oil on the coverslip can be easily removed with a rag moistened in xylol or turpentine.

[1] A solid culture, e.g., on gelatine or agar, may be used, in which case a drop of sterilised distilled water is first placed in the centre of the coverslip, and a little of the culture is rubbed up with the water to make an emulsion.

2. A hanging drop, being hemispherical in shape, is of different depths at different distances, near or far, from the periphery at which it is examined.

3. Animate and inanimate matter in finely divided condition, for different reasons, both tend to escape to the periphery of a hanging drop, which therefore is always the best place to examine first.

To find the edge of the hanging drop.—The danger of over-focussing, and so suddenly breaking the coverslip, is so great that the following method may be adopted in every case with advantage.

With the $\frac{2}{3}$-inch objective examine the edge of the drop, using the iris diaphragm to cut off a little of the light and bring the edge into better relief. It will be seen that immediately surrounding the sharp margin of the drop is an area of minute globules of watery vapour—which for brevity may be called 'dew'—and which almost invariably forms on the under surface of the coverslip soon after it is fixed in position. This dew is very much easier to focus than the edge of the drop itself with the oil-immersion lens. The direction of the dew, to the right or left of that portion of the edge of the drop under examination, is noted, and the $\frac{2}{3}$-inch objective replaced by the $\frac{1}{12}$-inch immersion lens. The oil being dropped on as usual, the dew is carefully focussed, and, *without altering the focus*, the slide is gently pushed in the direction, to the right or left, in which the edge of the drop is known to lie. If this rule is observed, there is not the slightest difficulty in examining a hanging drop, and coverslips need never be broken.[1] The hanging drop is examined first at its edge and then in its more central portion, the shape, length, motility, and grouping of the organisms (if any) noted, the focus of course being altered for the varying depths of the drop.

A hanging drop may be kept for from thirty-six to forty-eight hours, or even longer, for examination purposes, if a sufficiently large quantity of fluid, and plenty of vaseline for sealing the edge of the coverslip, be used.

To clean a hanging-drop preparation, when finished with.—With fine forceps push the coverslip towards the edge of the slide; now lift it up by one edge, and burn in case of a virulent culture. Otherwise the coverslip may be thrown away; or it may be cleaned and then boiled in nitric acid (p. 13).

Impression preparations (the so-called 'Klatschpräparate' of the Germans) are made by placing a clean coverslip carefully down on the surface of a selected colony in a 'plate' (petri-dish) cultivation.

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[1] Instead of a $\frac{1}{12}$-inch oil immersion, a $\frac{1}{6}$-inch objective, with a No. 4 eyepiece, may also be used.

gently pressing the coverslip so as to make the growth adhere to it without breaking up the colony, and lifting it up gradually by means of a fine pair of forceps.

The colony and adherent gelatine (or agar) on the coverslip are dried in the air, or high up above the flame, so as not to melt the medium, and fixed by rapidly passing the coverslip three times through the flame, or by means of absolute alcohol kept on for two minutes and then poured off. The coverslip is then stained in the usual way, preferably by carbol-fuchsin kept on for two minutes, and washed thoroughly. Spores, when present, can also be stained for by Möller's method (see fig. 75, p. 137).

The special advantage of impression preparations is that the minute structure of the colonies can be made out, which is impossible when examining the plates with the low power.

Further, in the case of cultures readily involuting or degenerating—e.g. *Vibrio choleræ asiaticæ*—plating and then making impression preparations is a good way, and sometimes the only way, to obtain undegenerate organisms.

To measure a microscopic object such as a micro-organism, an eyepiece micrometer and a stage micrometer (figs. 21 and 22) are required. The eyepiece micrometer is a circular disc of glass, fitting into a brass rim, with a scale of divisions, each of which actually measures $\frac{1}{10}$ mm.; but, as we shall see later, the length of each such division is estimated by comparison with that of a division of the stage micrometer. This is merely a glass slide, in the centre of which a series of very fine divisions have been engraved, the divisions being, respectively, equal to tenths and hundredths of a millimetre.

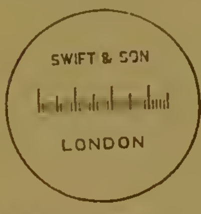

Fig. 21.—Eyepiece micrometer

The eyepiece micrometer is inserted into the eyepiece[1] after the top lens has been removed. On replacing this lens the scale of the micrometer is seen to be magnified, the degree varying with the number of the ocular or eyepiece, but always being much less enlarged, of course, than is the scale of the stage micrometer when viewed with both ocular and object glass. The stage micrometer scale is focussed, and the ocular adjusted, so that the two scales are exactly superposed. An estimate is then made of the number of divisions in the eyepiece micrometer which correspond to one of the smaller divisions of the stage micrometer, which we know measures $\frac{1}{100}$ mm. If five divisions of the eyepiece scale exactly coincide with one of these divisions on the stage micrometer, *using the same ocular and objective*, each of the former is equivalent to $\frac{1}{500}$ mm. or ~~$\frac{1}{2}$~~ μ, the letter 'μ' indicating the standard of length generally employed in measuring microscopic objects, the *micron*, which is the thousandth part of a millimetre in length (about $\frac{1}{25000}$th of an inch).

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[1] The eyepiece micrometer rests on a stop in the ocular, and as slight adjustment of the stop is generally necessary in order to focus the scale clearly, this should be done by the maker at the time of buying the micrometer.

A record is then made that, say, with No. 2 ocular and $\frac{1}{12}$-inch oil immersion (with the tube of the microscope drawn out, or not, as the case may be), one division of the eyepiece micrometer is equivalent to ~~0·5~~ μ.

Errata

Page 33, line 3, *for* $\frac{1}{2}$ μ *read* 2 μ
" " 10, " 0·5 μ " 2 μ

is focussed in the usual way, and the number of divisions in the eyepiece micrometer crossed by a given bacillus will be a measure of its length, the equivalent of each such division having already been estimated.

To sterilise a culture.—It is often desirable to sterilise a culture, or to fix it permanently, when it has reached a certain stage of development. The simplest way to do this is to remove the cork from a bottle of formalin (a 40 per cent. solution of formic aldehyde), and insert in its place the wool plug from the tube. The bottle should then be quickly inverted and replaced. The tube is now re-plugged with the wool (moistened with formalin) and covered with a rubber cap. Cultures may be kept for several years at least in this way, if evaporation is efficiently prevented by a tight-fitting cap.

PART II

SYSTEMATIC STUDY OF MICRO-ORGANISMS

NON-PATHOGENIC ORGANISMS

LESSON VI

THE CHROMOGENIC BACTERIA

A. **Bacillus prodigiosus**

I. Make *cultivations*:[1]

(i) Gelatine stab. Incubate at 20° C.

(ii) Agar-agar streak
(iii) Potato
Make three cultivations on each of these media, and incubate one of each at 37° C., one in *direct sunlight* at the temperature of the room, and the third in a dark chamber at the temperature of the room, e.g. any cupboard or closed drawer.

Observe the growths daily, and notice that the rich crimson colour is best developed at the lower temperature, and least at 37° C. Direct sunlight is also prejudicial to the formation of the colour; but, not acting continuously, the effect is not so striking as in the case where the culture is kept at 37° C.

II. *Stain coverslip preparations*[2] with, respectively,

(i) Löffler's methylene blue, for five minutes. Wash; dry, and mount in Canada balsam.

(ii) Carbol-fuchsin, for two minutes.
(iii) Anilin-water gentian-violet, for two minutes.
Wash well for ten to fifteen minutes at least. Dry and mount.

Owing to the sliminess of the growth in question, it is often difficult to obtain a thoroughly clean preparation, the mucoid material between the individual bacilli retaining the stain somewhat, so that the outline of the latter is not so well defined as it should be.

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[1] In each lesson the student is advised to make *all* the cultivations (A. B. C. &c.) before commencing to stain any of the coverslip preparations.

[2] For the general method of making and staining film preparations, see pp. 13-18.

To obviate the difficulty, after drying and fixing the coverslip preparation, held in cornet forceps, pour on acetic acid 20 per cent.; after ten minutes, pour off, wash thoroughly, dry between the folds of a pad of 'German blotting-paper' (p. 18), and proceed to stain in the ordinary way.

This is a useful method in the case of other slimy growths—such as that of *B. mallei*, *B. coli communis*, and for preparations made from stab cultures (e.g. glucose agar) of the anaërobes—to clear away some of the medium, which must always be more or less present with the organisms in such a case.

B. **Bacillus cyanogenus**

I. Make *cultivations*:

- (i) Gelatine stab. } Incubate at 18° to 20° C.[1]
- (ii) Gelatine streak. }
- (iii) Agar stab. }
- (iv) Agar streak. } Incubate at 37° C.
- (v) Potato. }
- (vi) Milk undergoing lactic acid fermentation; keep at from 15° to 18° C.

II. *Stain coverslips* with, respectively,

- (i) Löffler's blue }
- (ii) Carbol-fuchsin } for the times mentioned above.
- (iii) Anilin-water gentian-violet }

C. **Bacillus pyocyaneus**

I. Make *cultivations*:

- (i) Gelatine stab. Incubate.[1]
- (ii) Agar stab. Incubate.
- (iii) Agar streak.
- (iv) Potato. Make two cultures and keep at 37° C. After incubating at 37° C. for seventy-two to ninety-six hours, with sterile platinum needle exert gentle pressure on the growth in one tube, using the other for comparison. After a few minutes, sometimes almost instantaneously, the colour is seen to have changed from brown to green, the original colour reappearing in ten to thirty minutes ('Chameleon phenomenon' of Ernst).

II. *Stain coverslips*, as usual, with Löffler's blue, carbol-fuchsin, and anilin gentian-violet.

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[1] Henceforth, to avoid repetition in the directions, unless otherwise mentioned, all gelatine, or potato-gelatine, growths are to be incubated at 18° to 20° C., and growths on all other media are to be kept at 37° C.

Introductory Remarks

Commencing the systematic study of bacteriology from a purely practical point of view, micro-organisms may be broadly divided into those which are pathogenic and those which are non-pathogenic in their effects when inoculated into animals. As a general rule, only the pathogenic are considered of consequence by students of clinical medicine, as distinguished from those engaged in public health, for whom the knowledge of the ordinary organisms occurring in the air, soil, and water, whether pathogenic or otherwise, is important. But, as we shall presently see, the non-pathogenic organisms (so called) have an interest also for the clinical investigator, not merely because a lack of knowledge in this direction might cause him to confuse the one with the other under the microscope, but on account of other well-established relations between the two classes. For instance, a culture of typhoid bacilli which has lost its virulence may become pathogenic if, at the time of, or some time before, its inoculation into an animal, the products of an ordinarily harmless bacillus, such as the *Bacillus prodigiosus*, are also injected. Yeast ferments, also, have been used in the treatment of cancer (see p. 59).

A more scientific classification separates those organisms which live on dead matter and are known as '**saprophytes**' from those which thrive only on living matter, and are hence termed '**parasites.**' There are intermediate forms which are not strictly one or the other, being generally, for instance, saprophytic, but capable, under certain circumstances, of taking on a parasitic existence; and *vice versâ*. Such an organism as the former is termed a '**facultative parasite.**' If strictly parasitic, the fact may be indicated by prefixing the adjective '**obligate**' to it. So for the aërobes and anaërobes (see Lesson XX.).

Most of the organisms we shall study are called **aërobes**, requiring to live in an atmosphere containing oxygen. Certain of these, however, can also continue to grow in the absence, more or less complete, of oxygen. Such aërobes are said to be '**facultative anaërobes.**'

Anaërobes are organisms preferring to grow in an atmosphere deprived of oxygen. Those which *cannot* live in an atmosphere of oxygen, *i.e.* are strictly anaerobic, are called '**obligate anaërobes.**' Those which, generally speaking, thrive best away from the air, but which can exist in contact therewith, might be spoken of as '**facultative aërobes.**'

At the present time, 'bacterium' is a general term applied to those lower fungi (excluding yeasts and moulds) which form the majority of micro-organisms, pathogenic and non-pathogenic. Their vegetable nature is now no longer questioned; and, indeed, from their close relationship to the Algæ they have been called 'Achlorophyllous Thallophytes.' By botanists they are included in the group of *Schizomycetes*, or fission-fungi, from their usual mode of multiplication—viz. by division at right angles to their general direction. Under certain circumstances, however, spore formation may occur, either as well as, or in place of, multiplication by fission.

The term 'bacterium' was formerly used in a sense practically synonymous with 'bacillus,' and this application of the word may be illustrated by the name of one of the commonest organisms found in the intestine, the *Bacillus coli communis*, which is still frequently called *Bacterium coli commune*.

For the purposes of diagnosis only, most of these bacteria may be placed in one of the following **three groups**, according to their shape :

1. **Bacillus.**—A *bacillus* is a cylindrical or rod-shaped organism, of varying length, and sometimes elongated to form a thread (fig. 93) showing signs of segmentation. Spore formation may also be visible. One or both extremities of the bacillus may be rounded, or flattened, or concave (e.g. *B. anthracis*), or pointed.

Long, very thin, and straight, or slightly curved, unbranched filaments, in which segmentation is indistinct, occur in the mouth in large numbers, especially where tartar has collected or caries is present. They have long been known by the name of *Leptothrix buccalis* (fig. 23), several species of which are described. (In association with other organisms, cocci, &c., they are thought to be a common cause of dental caries.)

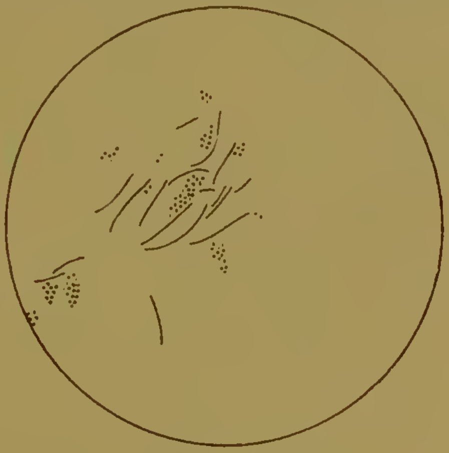

FIG. 23.—LEPTOTHRIX BUCCALIS FILAMENTS AND STAPHYLOCOCCI, IN AN AGAR-AGAR CULTURE FROM THE MOUTH. × 530

Similar, but thicker, threads are found in marshy districts, (*Beggiatoa alba*, &c.).

Threads showing branchings are seen in the case of the *Streptothrix* filaments of *Actinomycosis* (fig. 108) and *Madura-foot*; another example of this condition occurs in the case of *Cladothrix dichotoma*, abounding in water of all kinds, running or stagnant, fresh or brackish, especially when there is a large percentage of organic matter present, when it may form flocculent white masses.

2. **Spirillum.**—This group is conveniently made to include all well-

curved rods, the short simple curves being known as **vibrios**, and, sometimes, as **comma-shaped bacilli** (the German comma)—e.g. the *Vibrio choleræ Asiaticæ*, or *Koch's comma bacillus* (fig. 103). When the rod is longer and spirally twisted, the true **spirillum** is seen (fig. 30). Still longer twisted threads, bent in various directions, are known as **spirochæte** forms (fig. 30).

3. **Coccus,** or **micrococcus.**—**Cocci** are spheres of varying size, but usually very minute, occurring singly; or in pairs (**diplococci**); or like a string of beads, more or less curved (**streptococci**); or in masses grouped like a bunch of grapes (**staphylococci**); or in fours, resulting from division of the sphere in two directions, but in one plane only (**tetrads**); or in cubes or **packets**, resulting from division of the sphere in three directions, so characteristically seen in the case of sarcinæ (see p. 46).

As the result of their vital activity, certain organisms known as **chromogenic bacteria** produce pigment amongst other products of their metabolism (see p. 42). Many of them are saprophytes, and we shall study a few examples of this class in the systematic investigation of the non-pathogenic and pathogenic micro-organisms which we are about to commence.

NON-PATHOGENIC ORGANISMS CHROMOGENIC BACTERIA

Bacillus prodigiosus.—From the remarkably striking colours seen in the growth of this organism it has been given many names, popularly, 'bleeding host,' 'blood rain,' 'bloody sweat,' &c.

Occurrence.—In the air and on bread (when it has been called 'bleeding bread'), paste, boiled rice, and other starch-containing bodies. It may appear in large quantities, and so persistently in the bread supplies as to produce quite an epidemic, as in the well-known case of certain Parisian barracks in 1843. This organism has been found in the water by Tils and Percy Frankland. The colour of the growth varies from a bright red, often with a metallic lustre, especially in old cultures, to a rich carmine or port-wine tint. A low temperature is the most favourable for its production, as when kept at 37° C. the cultures lose the power of pigment formation to a large extent. Returning such cultures to the lower temperature, especially if they are transferred to potato, restores the colour. If kept for a series of generations on agar at the body temperature, the power of forming pigment may, however, be almost completely lost.

Cultivations.—*Gelatine stab.*—A crimson growth rapidly appears along the track of the needle, and liquefaction, commencing from the surface, quickly spreads from top to bottom, owing to the peptonisation of the medium as the result of the vital activity of the organism.

The liquefied gelatine is viscid and of a beautiful crimson colour throughout, the most superficial layer to a depth of about $\frac{1}{16}$ inch being of a deeper and richer hue than the rest of the medium below.

Gelatine plates.—Translucent grey colonies rapidly appear, and early liquefaction occurs with the formation of the characteristic colour in the inner, more central part of the colony. The surface colonies, which are irregular in outline compared with the deeper and more circumscribed ones, soon fuse into one another from the rapidly progressing liquefaction.

Agar-agar streak.—A smooth moist growth rapidly appears, the tint varying from brick-red to crimson, if kept at the room temperature. When incubated at 37° C. the colour is white, with a faint pink tinge, the higher temperature being unfavourable to the formation of the red pigment.

Potato.—A rich crimson-coloured growth rapidly forms at the temperature of the room or a little higher; often it is brick-red, and has the peculiar metallic lustre generally seen when anilin dyes are viewed obliquely. At the body temperature, or if kept in strong sunlight, the culture grows well, but does not acquire its characteristic colour, remaining white or pinkish-white, the crimson tint appearing when the tube is kept in the dark at the room temperature.

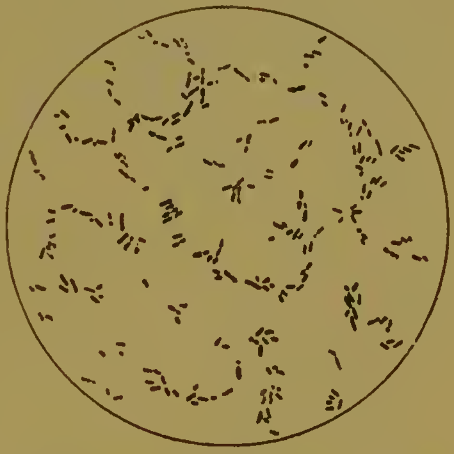

FIG. 24.—BACILLUS PRODIGIOSUS. ×925
From a culture two days old.

Milk, especially when kept at the body temperature or a little higher, is curdled, with the formation of lactic acid, from the lactose present, and the precipitation of the casein.

Schottelius and Wood (quoted by Sims Woodhead) comment on the probable relation between the last-mentioned fact and the loss of pigment-forming power at the higher temperatures, and consider that the energy expended in the production of pigment at the lower temperature is, at the higher and less favourable temperature, utilised for the production of other products, of which lactic acid is one.

Under the microscope, the organism is seen to be either a very short rod with rounded ends, sometimes arranged to form threads, or a short oval bacillus, often in pairs. In length they are 0·5 to 1 or even 1·7 μ, their width being from 0·5 to 1 μ. They are non-motile; no spore formation is known. The pigment is soluble in alcohol, but is insoluble in water. The question of colour formation, generally, by chromogenic organisms, will be referred to at the end of the chapter (p. 42). *B. prodigiosus* enters into the manufacture of Coley's fluid (p. 111). For the employment of this non-pathogenic organism in order to obtain virulent cultures of typhoid bacilli, &c., see p. 181.

Bacillus cyanogenus is the organism of blue milk, from which it was originally isolated. A somewhat similar organism, having the same name, has also been isolated from sewage, but does not produce blue milk.

Cultivations.—*Gelatine streak.*—A dirty greyish-white moist growth appears, which does not liquefy the gelatine. Extending from the sides of the growth, and also backwards into the medium, a greyish-brown coloration is visible. The tint varies, being sometimes of almost a steel-blue colour, dark brown, or even black.

Gelatine stabs show the same tinting of the medium, the surface growth being raised, moist, and greyish-white.

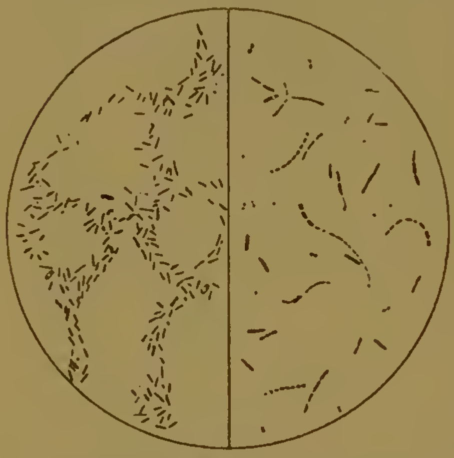

FIG. 25.—BACILLUS CYANOGENUS. × 530

From a culture three weeks old

From a culture two months old

Agar-agar streak.—The surface growth is often of a dirty grey colour.

In the depths, and *in stab cultures*, a dark brown, black, or sometimes a blue-black, coloration of the medium is seen.

Potato.—The growth may at first be yellowish, but soon a greenish-black tint is given to the medium around.

In *milk*, when alkaline, a slate colour is formed if kept at 15 to 18° C. In the presence of lactic acid fermentation a blue colour is produced. Keeping the milk at 37° C. prevents the formation of this colour, which may take a considerable time to develop.

Under the microscope, the organism is seen to present a great variety of shape. It occurs as rounded cocci-like bodies; or as short rods, sometimes encapsuled and collected into zooglea-like masses; or as medium-sized rods with rounded ends containing a spore in the middle: if the spore develops terminally, the bacillus is more or less club-shaped. Longer, irregularly swollen, involution forms are also met with. The length has been stated to vary from 1 to 4 μ, and its breadth from 0·3 to 0·5 μ. It is very motile, except when in the zooglea-like masses.

Bacillus pyocyaneus is the organism of blue, or greenish-blue, pus. This bacillus, though pathogenic when injected subcutaneously, or intraperitoneally, into guinea pigs and rabbits,[1] may be conveniently treated of here.

Cultivations.—*Gelatine stab.*—Rapid liquefaction of the medium occurs; the upper fluid portion, especially where in contact with the air, being of a beautiful green tint, as seen both by reflected and by transmitted light.

Agar-agar stab.—The whole of the medium rapidly turns green or greenish-blue, which becomes darker with age. The surface growth is moist and white, with a well-marked bluish-green tint; so for the *agar streak* cultivation.

Potato.—A pale, reddish-brown, moist growth is seen, often also a greenish tint is added. The green tint becomes still more obvious on the addition of ammonia, whilst the addition of a little acid accentuates the red colour.

If the growth on potato after from seventy-two to ninety-six hours' incubation at 37° C. is gently pressed with a sterile platinum needle, the red-brown colour is seen to change to green, the original colour reappearing in from ten to thirty minutes. This is known as the 'Chameleon phenomenon' of Ernst, who has paid much attention to this organism (Abbott). The phenomenon, however, does not always appear, its presence or absence depending probably on the great differences observable in potatoes as regards their reaction.

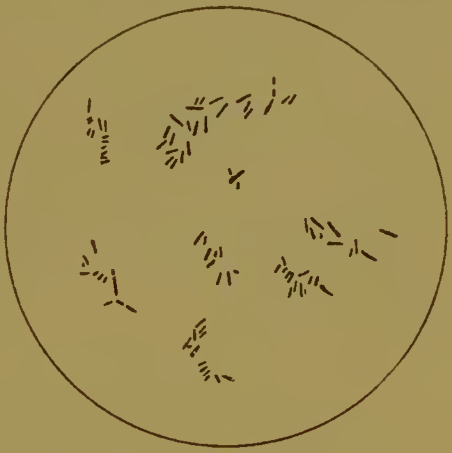

FIG. 26.—BACILLUS PYOCYANEUS. × 925

Under the microscope, the organism is seen to be a slender rod-shaped bacillus, with rounded extremities. Its length varies, being sometimes hardly longer than it is broad, so that it might be mistaken for a coccus; whilst in old cultures fairly long forms are seen, the majority, however, being short thin rods about the same length, but slightly thicker than in the case of the bacillus of mouse septicæmia (p. 215). Spore formation appears to be present, though this is denied by Abbott. It is very motile, and owes its motility to the presence of a single flagellum at one end.

The virulence of this organism[1] for rabbits and guinea pigs passes off the longer it has been subcultured; but it may be restored by successive inoculation through a series of animals from one to another.

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[1] Symmers, and others, have recently brought forward evidence to prove that *B. pyocyaneus* may be pathogenic for man also, and not merely saprophytic, as hitherto believed.

The subcutaneous injection of 1 c.cm. of a virulent culture may be followed by death in less than twenty-four hours (eighteen to thirty-six hours being the average time), with blood-stained œdematous infiltration, or even well-marked suppuration, extending from the point of inoculation. A general septicæmic condition may be produced, the blood and tissues containing the bacilli in large numbers. Smaller doses may be followed by merely an abscess at the seat of injection. By repeated sub-fatal doses an animal may be made immune to the organism, and its serum, when injected into another animal, is both prophylactic if administered prior to inoculating the organism, and curative when given in the proper proportions after the disease has been introduced.

The serum, moreover, has the same power of paralysing the activity and agglutinating the bacilli in a fresh broth culture of *B. pyocyaneus* as in the now well-known Widal's sero-diagnosis test for typhoid (see p. 183, *et seq.*), so that they fall to the bottom of the test-tube of serum in which they are grown, or with which they are mixed, instead of causing a uniform turbidity, as happens when they are grown in ordinary healthy serum; and, under the microscope, a hanging drop shows clumps of motionless organisms everywhere.

A further interesting fact about this organism has been established. Inoculation with an active culture of *B. pyocyaneus* shortly after the injection of a virulent culture of *B. anthracis* will counteract the fatal effects of the latter.

A similar result follows the use of sterilised cultures, the antagonistic effects being therefore due to the chemical products of the bacilli (Sims Woodhead and Cartwright Wood).

From cultures of *B. pyocyaneus*, and from the blue pus, or dressings stained therewith, the product called *pyocyanin* has been extracted by means of chloroform, from which bluish-green needle-shaped crystals separate out.

The formation of colours by micro-organisms.—Briefly, we may say that in most cases the pigment exists in the sheath rather than in the protoplasm of the organism; and owing to the interaction between the organism and the surrounding medium, whether natural or artificial, this pigmentation may spread far and wide into the latter, as we have already seen. The beautiful rainbow-like tints and other changes in colour which putrescent matter undergoes must have been observed by every one. These effects are associated with the presence of a great variety of micro-organisms, yeasts and moulds, different kinds of sarcinæ, bacilli, &c. The formation of pigment, like that of gas or acid, in the case of other organisms—e.g. *B. coli communis*—is simply a product of their metabolism. Some organisms have an affinity for any iron in their vicinity, and, in the form of oxide, this metal is sometimes stored up in their sheath. The *Cladothrix dichotoma*, found in water, is an instance in point. Other organisms have a special affinity for sulphur, which, arising from the decomposition (into sulphuretted hydrogen) of the sulphates universally present in water, soil, &c., may become stored up as granules in the protoplasm of the organism itself, as in the well-known case of *Beggiatoa*.

We may suppose that in this way sulphur and iron are brought into connection with one another, and that the pigment is essentially iron sulphide in combination with an organic base. This mode of pigment formation may be compared with the various colours in precious stones, whch, though so different in appearance, are often almost identical in chemical composition, their various tints resulting from similar combinations of iron with sulphur, oxygen, &c. There is reason to suppose, however, that pigments may also be formed apart from the presence of iron, arising from the metabolism of purely albuminoid substances.[1]

LESSON VII

THE CHROMOGENIC BACTERIA (*continued*)

A. **Bacillus fluorescens liquefaciens**
B. **Bacillus fluorescens non-liquefaciens**

I. *Cultivations of* A *and* B : (i) Gelatine stab.[2]
,, *of* B *only* : (ii) Gelatine streak.
,, *of* A *and* B : (iii) Agar stab.
,, *of* A *and* B : (iv) Agar streak.
II. *Stain coverslips* of A and B with
(i) Carbol-fuchsin.
(ii) Anilin-water gentian-violet.

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[1] For further details the reader is referred to the very interesting account in Dr. Sims Woodhead's *Bacteria and their Products*, to which I must acknowledge my indebtedness in the above *résumé*.

[2] See footnote, p. 35.

C. Micrococcus agilis

I. *Cultivations*: (i) Gelatine stab.
(ii) Gelatine streak.
(iii) Agar streak.
(iv) Potato.

II. *Stain coverslips* with
(i) Carbol-fuchsin.
(ii) Löffler's methylene blue.

III. *Make a hanging drop* from a fresh culture—broth, by preference[1]—and examine it under $\frac{1}{12}$-inch oil immersion. Notice the single cocci, cocci in twos and fours (diplococci and tetrads) and chains, moving actively, the movement being rotatory as well as from place to place.

D. Sarcina lutea

I. *Cultivations*: (i) Gelatine stab.
(ii) Agar streak.

II. *Stain a coverslip* with carbol-fuchsin.

E. Spirillum rubrum

I. *Cultivations*: (i) Gelatine stab.
(ii) Gelatine streak.
(iii) Agar streak.
(iv) Broth.

II. *Stain coverslip preparations* from a solid and broth culture, respectively, with carbol-fuchsin.

Bacillus fluorescens liquefaciens

Bacillus fluorescens liquefaciens.—According to Frankland, this organism, or slight variations of it, is perhaps more frequently found in water than any other form. It is also found in putrid infusions.

Cultivations.—*Gelatine stab.*—Along the track of the needle the growth is seen as a white filament. In the upper part a cup-shaped region of liquefaction is soon seen, which is yellow when observed by transmitted, and green by reflected light, producing a characteristic fluorescence. At the bottom of the tube is a deposit of dense white growth.

Agar streak.—The growth is of a dirty white colour, with a greenish-yellow coloration of the medium.

Potato.—A brownish layer is produced.

Under the microscope are seen (fig. 27, A) very short rods with rounded ends, 1 to 1·5 μ long, by 0·5 μ broad. It is motile, but no spore formation has been observed.

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[1] *Note for the teacher.*—It is convenient to have a fresh broth culture ready for the class on the day it is required.

Bacillus fluorescens non-liquefaciens.—This organism occurs in water.

Cultivations.—*Gelatine streak.*—A thin, fluorescent, greenish-white film is produced. In gelatine plates the surface colonies form characteristic 'fern-leaf' patterns, and are somewhat opalescent; by transmitted light, they are yellow; by reflected light, they are greenish. The gelatine is not liquefied.

Gelatine stab.—The growth is very slight, compared with that seen in the case of the preceding organism.

Potato.—The growth appears rapidly as a brownish layer, and the surrounding surface then assumes a bluish-grey tint.

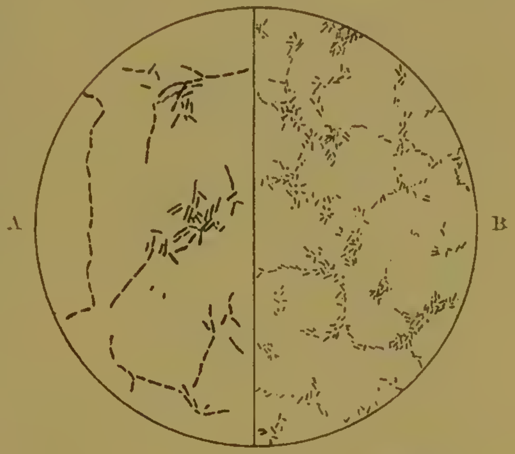

Fig. 27.—A, Bacillus fluorescens liquefaciens. B, Bacillus fluorescens non-liquefaciens. × 530

Under the microscope, short, and very thin rods with rounded ends, are seen (fig. 27, B). It is non-motile, and no spores have been seen. Several other fluorescent bacilli are known.

Micrococcus agilis.—This also occurs in water.

Cultivations.—*Gelatine stab.*—The organism spreads as a pink growth along the track of the needle, and only slowly produces liquefaction (about three weeks or a month).

The *surface growth on gelatine,* on *agar-agar,* and *potato,* is of a beautiful pink colour. A variety of this organism, *Micrococcus agilis citreus,* also present in water, is identical under the microscope, but its cultures are of a lemon-yellow colour.

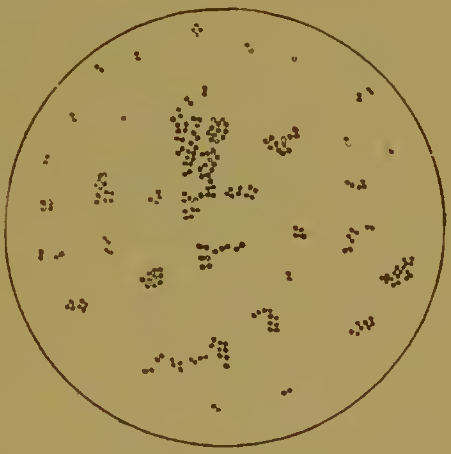

Fig. 28.—Micrococcus agilis citreus. × 530

From a culture two days old.

Under the microscope, cocci, singly and in pairs, especially the latter, and in groups, are seen. Their diameter is about 1 μ, and they exhibit well-marked motility, the pairs and groups of cocci rotating as they move from place to place. The single flagellum attached to each coccus is very fine, and has been estimated to be four or five times the length of the organism.

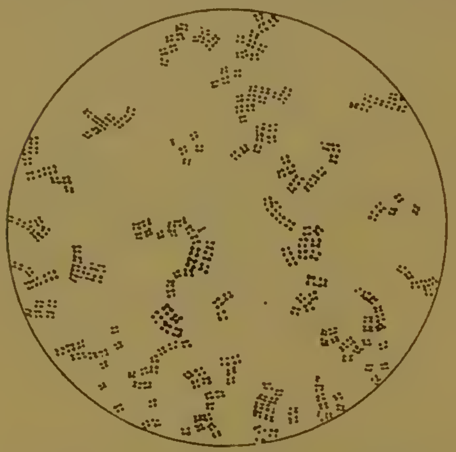

FIG. 29.—SARCINA LUTEA (AGAR-AGAR CULTURE). NO 'PACKETS,' OR CUBES, ARE SHOWN. ×530

Sarcina lutea.—This occurs in the air; and a similar organism, the *sarcina ventriculi*, is found in the vomit in cases of simple dilatation of the stomach and in pyloric obstruction due to carcinoma pylori.

Cultivations.—*Gelatine stab.*—The growth along the track of the needle is of a paler tint than that on the surface, which is of a beautiful canary-yellow colour. Liquefaction of the gelatine[1] occurs.

Agar streak.—The growth is similar in colour.

Under the microscope, the organisms are seen to occur singly, in pairs, in fours, and in cubes, which present the characteristic appearance of corded bales of cotton. These cubes are often well seen in the liquefying colonies on gelatine plates, using the $\frac{2}{3}$-inch objective. But perhaps they are best seen in vomited matter in the cases mentioned above.

These 'packets,' or cubes, are formed by multiplication of the cocci in three directions (see p. 38).

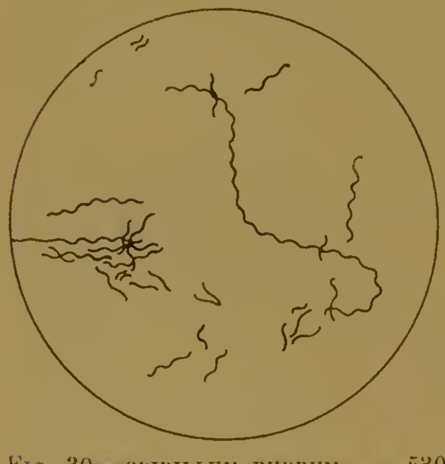

FIG. 30.—SPIRILLUM RUBRUM. ×530

Spirillum rubrum. This was first isolated from the putrefying tissues of a mouse dying of mouse septicæmia. It is a typical spirillum, and may therefore be conveniently used to represent the *Spirillum Obermeieri*, the organism present in the blood of patients suffering from relapsing fever.

Cultivations. *Gelatine stab.*—The growth along the track of the needle consists of fine granules of a beautiful crimson colour, whilst that on the free surface is almost colourless. There is no liquefaction.

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[1] *Sarcina ventriculi* and *Sarcina pulmonum* (which is found in phthisical sputum) do not liquefy gelatine.

Gelatine streak.—The growth is white and moist. On holding the tube up to the light the deeper part of the growth may be seen to be of a light pink tint.

Agar-agar streak.—The growth is similar to that on gelatine, but the pink tint is generally more pronounced.

Broth.—The growth sinks to the bottom, and is of a pink colour.

Under the microscope, spirally twisted rods are seen. Some of these form long threads, which bend at a right angle in one or more places (*spirochæte* forms). In broth, longer spiral threads are formed than on the solid media. Growth is always somewhat slow.

The organism is very motile, and has a pair of long flagella at either end, which may be stained by the ordinary method for flagella (see p. 169, *et seq.*).

It readily undergoes degeneration or '**involution,**' as it is otherwise known. Under these circumstances, the organism is thicker, the spirals are less marked,—the coils being apparently opened out—and vacuoles are seen; lastly, the involuted organism takes the stain less intensely than when in its normal condition. The appearance of the cultures, however, remains typical.

LESSON VIII

A. **Bacillus subtilis**
B. **Bacillus megaterium**
C. **Bacillus mycoides**

I. *Cultivations* of A, B, and C, on

(i) Gelatine stab.[1] (ii) Agar-agar streak. (iii) Broth.

Compare each of these three sets of cultures.

II. Stain *coverslip preparations* of A, B, and C with carbol-fuchsin.

III. Stain A, B, and C for *spores* by the following method :

Moller's method for staining spores.—1. Make a thin film preparation on an absolutely clean coverslip ; dry, and fix as usual. Hold in cornet forceps.

2. Pour on absolute alcohol.[2] After two minutes, drain. Again dry, and fix in the flame.

3. Pour on chloroform. After two minutes (when the fluid will have become cloudy), drain, dry, and fix in the flame, as there is considerable tendency for the film to get loosened during this stage.

---

[1] See footnote, p. 35.

[2] Good methylated spirit may be used instead of absolute alcohol.

4. Pour on chromic acid 5 per cent. After two minutes, wash the coverslip, gently but thoroughly, to get rid of the yellow colour. Dry and fix in flame.

5. Filter carbol-fuchsin on to the film, and warm till it steams. Place the coverslip in the paraffin cupboard, or warm incubator, for ten to fifteen minutes, after which wash away the excess of stain.

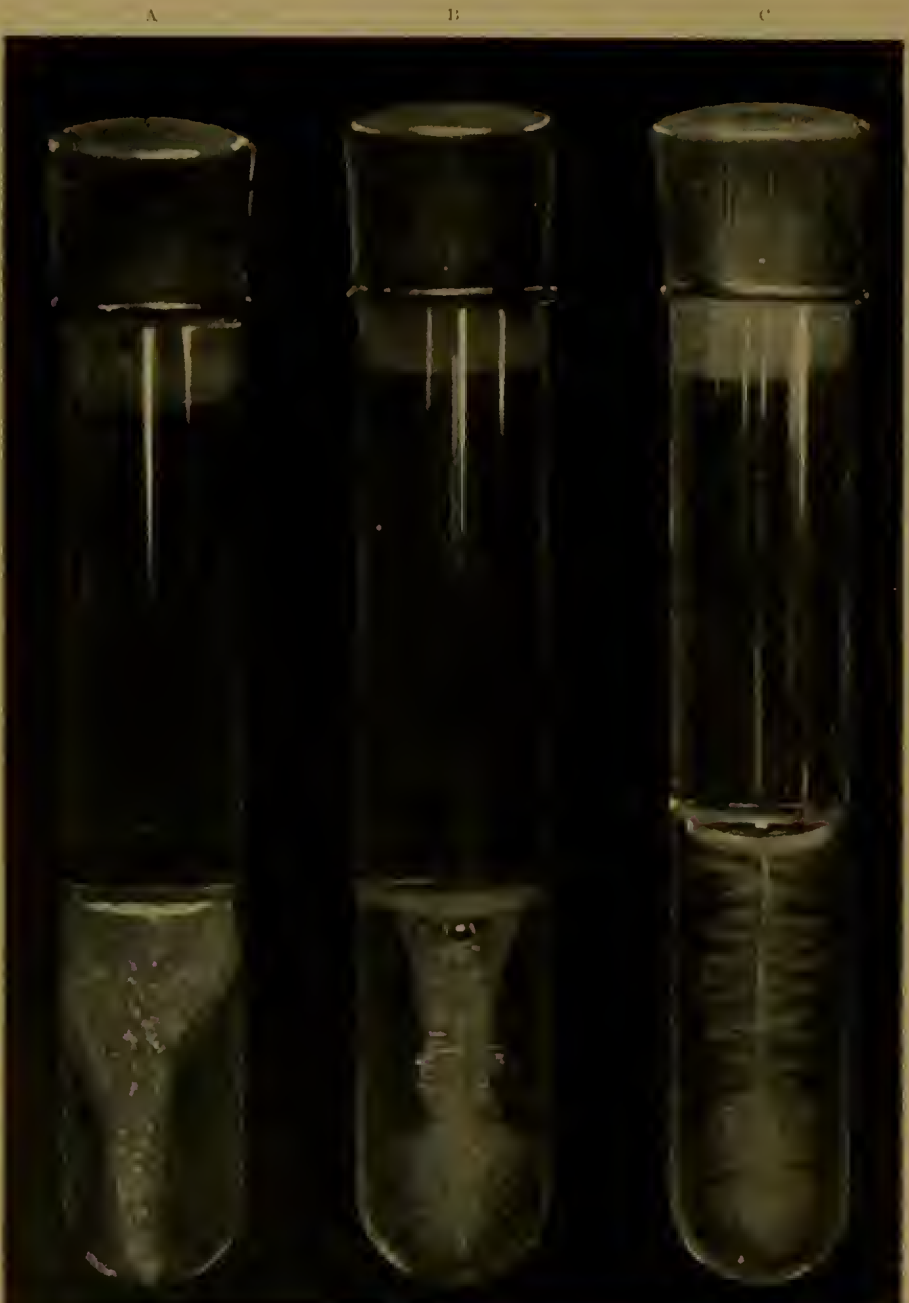

FIG. 31.—GELATINE STAB CULTURES (4 DAYS OLD) OF

A. BACILLUS SUBTILIS B. BACILLUS MEGATERIUM C. BACILLUS MYCOIDES

6. Decolorise with 5 per cent. sulphuric acid, in a watch-glass; but the coverslip must only just be dipped in and out, and *at once* well washed in water; otherwise the spores may also be decolorised. This is the critical part of the method.

7. Dry the film, and counterstain in Löffler's blue for five minutes. Wash well. If no blue colour is visible, counterstain again until the film is seen to be of a bluish tint. Wash, dry, and mount. The spores will be stained a beautiful red colour and the bacilli blue.

IV. *Make a hanging drop* from the broth cultures of A, B, and C, after incubating them overnight at 37° C. Observe the motility of the organisms in each case.

Bacillus subtilis

Bacillus subtilis occurs almost universally, and, together with its spores, is especially abundant in packing material, in straw and hay; hence the popular name of it, 'the hay bacillus,' of which there appear to be several varieties.

Cultivations.—*Gelatine stab.*—A white growth is seen along the needle track, and the gelatine is rapidly liquefied (fig. 31, A), the growth sinks to the bottom, and a wrinkled scum is formed on the surface. As liquefaction proceeds, this first scum also sinks to the bottom and is replaced by a second. Frequently, however, liquefaction is slower than this, and from the track of the needle horizontal branches extend into the medium, producing a fine feathery growth. After a while, this appearance may be slowly replaced by the liquefying gelatine. This difference as to liquefying power seems to point to the existence of varieties of the organism.

Agar-agar streak.—The cultivation is very characteristic. It is opaque, white, and moist at first, with well-defined edges (fig. 32, A). As the growth becomes older its surface becomes much dryer and wrinkled, especially at the bottom of the tube, when the wrinkling often assumes a light fawn colour and may somewhat resemble a badly developed culture of *B. tuberculosis.*

Broth.—Turbidity rapidly sets in after inoculation at 37° C. A much-wrinkled dry white scum on the surface is characteristic of this organism.

Potato.—The growth is moist and white. Spore formation is very rapid on this medium.

Under the microscope, the growth is seen (fig. 33) to consist of rods, short and long, with rounded ends, often arranged in chains, and containing oval spores.[1]

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[1] See general remarks on spores, at the end of this chapter (p. 52).

The whole interior of the bacillus may be occupied with the spore, which appears as a clear oval body, seeming to show bi-polar

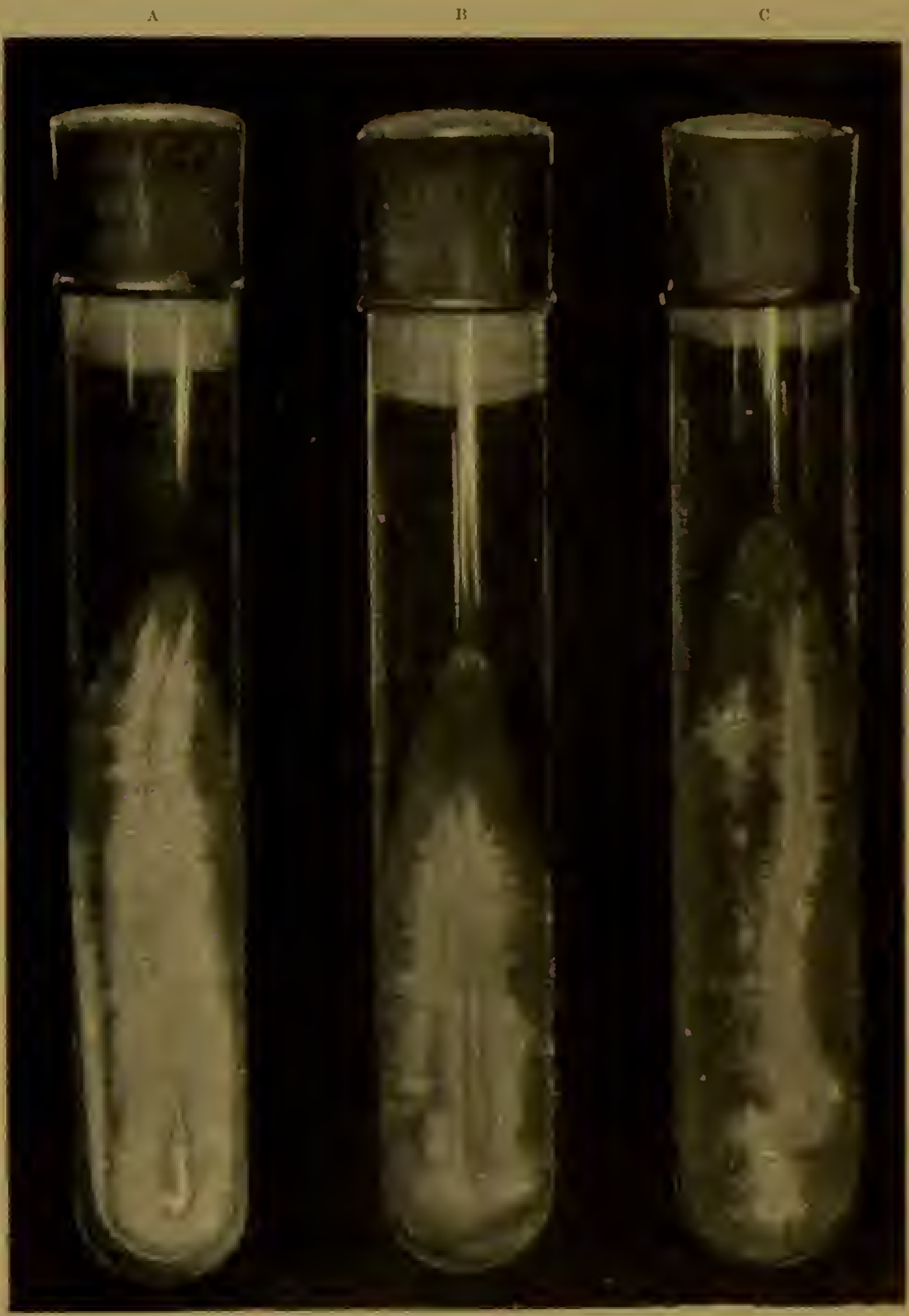

FIG. 32.—GLYCERINE AGAR-AGAR STREAK CULTURES.
A. BACILLUS SUBTILIS B. BACILLUS MEGATERIUM C. BACILLUS MYCOIDES

staining, the stained part being, however, the remains of the distended bacillus. When the spores are liberated from the bacilli they remain colourless if the simple stains have been used for the preparation. Möller's method stains the spores of a red colour, and the bacilli blue. The spores are large, and have been estimated to be 1·2 μ long, and 0·6 broad. They survive exposure for one hour to a temperature of 120° C.

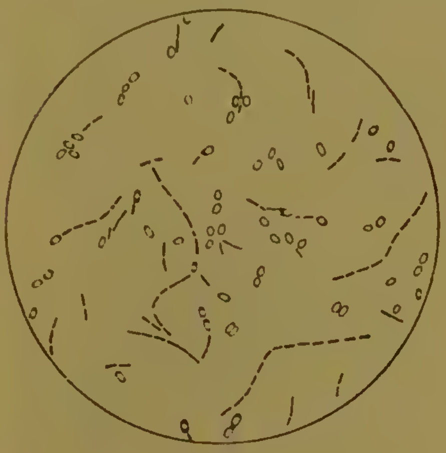

Fig. 33.—BACILLUS SUBTILIS, WITH NUMEROUS SPORES. × 925

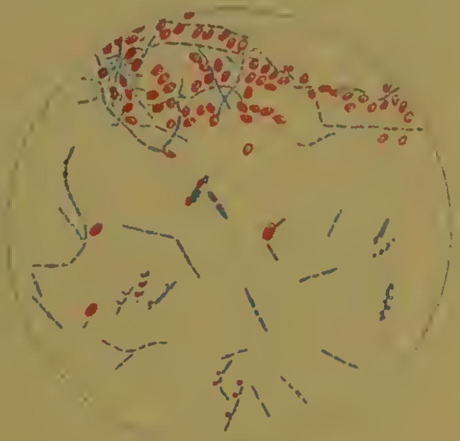

Fig. 34.—SPORE STAINING (MOLLER'S METHOD). × 925

In the upper part of the field, *Bacillus subtilis*: in the lower portion, *Bacillus megaterium*. Spores, red: bacilli, blue.

In a hanging drop the bacilli are seen to be very active in their movements.

Bacillus megaterium was first isolated from boiled cabbage leaves, and has been found in water.

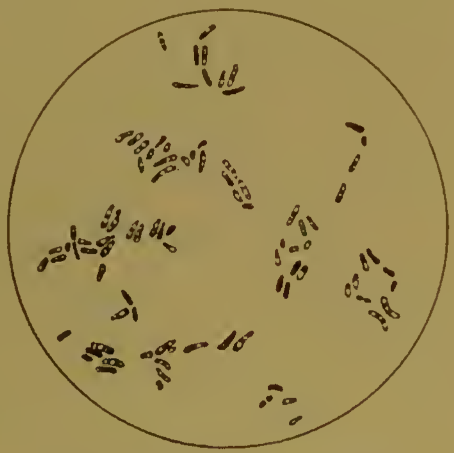

Fig. 35.—BACILLUS MEGATERIUM (EIGHTEEN DAYS OLD). × 925

Cultivations.—*Gelatine stab.* From the delicate growth along the needle track, fine hairlike filaments (fig. 31, B) are seen to spread out into the medium. These branches are unequal in size, but in other respects the growth is very similar to that of *B. mycoides*. Liquefaction rapidly ensues, the surface growth sinking gradually, as an opaque yellowish-white layer to the bottom of the tube.

Agar streak.—The growth is white and moist. From its edge fine filaments pass outwards (fig. 32, B).

Potato.—A yellowish-white growth appears, not nearly so profuse as in the case of *B. subtilis* and *B. mycoides*.

Under the microscope, a rod-shaped bacillus, frequently very long and thick, with rounded ends, is seen, often slightly curved, and containing black granules and spores (fig. 35).

Multiplication by fission also occurs. By the use of dehydrating agents separate divisions have been observed,[1] so that the bacillus then appears to be made up of a series of short rods aggregated together, each containing granules and a single spore. There is slight motility. The bacilli are provided with several flagella, sometimes as many as twelve. They are stained by Gram's method.

Bacillus mycoides.—This is one of the commonest organisms in the superficial parts of cultivated soil.

Cultivations.—*Gelatine stab.*—The growth spreads out from the track of the needle in filmy branches about equal in size, many of them becoming twisted together, so as to present a somewhat spiral appearance (fig. 31, c). Liquefaction is rapid.

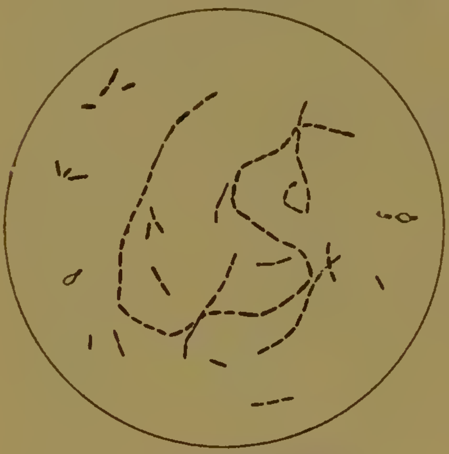

FIG. 36.—BACILLUS MYCOIDES. × 925

Agar-agar streak.—The growth is white and moist, with a fringe-like edge (fig. 32, c). Subsequently the surface becomes drier.

Broth.—Turbidity rapidly occurs. A white scum forms on the surface, and later on becomes of a brownish colour.

Potato.—A white slimy growth appears.

Under the microscope, rod-shaped organisms with rounded ends are seen, which are said to closely resemble *B. anthracis*. They are, however, decolorised by Gram's method. Their length is 1·5 to 2·5 μ, and breadth 0·75. Oval spores are present of about the same size as those of *B. subtilis*. The organism is motile.

Spores and spore formation.—Under favourable conditions bacteria multiply by a process of fission, hence the name *Schizo-mycetes*, or *Fission-fungi*, given to the class. Under unfavourable conditions, chief amongst which are to be mentioned defective nutrition generally, hyper-oxygenation or the contrary, and the accumulation of the metabolic products of the organism's vitality, multiplication by fission is checked and the formation of spores commences. These appear as minute, discrete, and highly refractile granules, which run together to form a single spore in each bacillus. Each spore consists of an outer gelatinous envelope, or *exosporium*, and an inner portion or *endosporium*.

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[1] De Bary, quoted by Crookshank (*Bacteriology and Infective Diseases*, p. 522).

Opposite views have been held as to the real nature of spore formation, whether it is, or is not, to be considered as an indication of commencing degeneration. However this may be, their structure explains both the great resisting power of spores to destructive agencies, drying, chemicals, &c., and the difficulty experienced in staining them by simple methods.

The central portion, or endosporium, stains readily only after the gelatinous envelope, or exosporium, has been specially prepared by maceration, &c., as in Möller's method, or by passing the film preparation rapidly and frequently through the flame so as to split the outer envelope sufficiently for the stain to reach the endosporium. This splitting of the sheath also appears to take place spontaneously, so as to allow the contents to elongate when the spore is about to germinate, and give origin to a bacillus.

It may be mentioned here that analogous difficulties, probably due to differences in chemical composition of the sheath, &c., are sometimes also met with in staining the *bacillus* itself, e.g. in the case of *B. tuberculosis*, *B. lepræ*, &c., and in attempting to demonstrate the *flagella* in the case of flagellate organisms, e.g. *B. typhosus*.

LESSON IX

YEASTS—FERMENTATION

A. **Saccharomyces cerevisiæ.**
B. **Torula rosea.**
C. **Torula nigra.**
D. **Saccharomyces (or, Oïdium) albicans.**

I. (*α*) Make *Cultivations* of A, B, and C on gelatine streak.
  (*β*) Make *Cultivations* of D on
    (i) Gelatine or agar streak, which contains little saccharine matter.
    (ii) Potato, which contains carbohydrate material in abundance.
    (iii) Glucose broth.
II. (*α*) Stain *coverslips* of A, B, and C with carbol-fuchsin.
  (*β*) Stain *coverslips* of D, grown in the sugar-broth, and on the media containing little or no saccharine material.

The sugar-broth preparation will present the appearance of ordinary yeast; the others also contain numerous filaments, which may be welded into a feltwork or mycelium, thus resembling a mould, in which class the fungus was formerly included.

Yeasts, putrefactive organisms, and moulds.—Having dealt with some of the non-pathogenic bacteria, and before passing on to those more generally spoken of as pathogenic, it will be useful to consider in this and the following lessons the yeasts, putrefactive organisms, and moulds, though a good many of the last mentioned are also recognised to be pathogenic in their effects.

Yeasts, moulds, and bacteria generally, are allied to the *algæ*, but being destitute of green colouring matter may be included under the common term *Achlorophyllous thallophytes*.

Bacteria are distinguished, principally, by their very minute size from yeasts. Multiplication by budding, or *gemmation* (fig. 37), is characteristic of yeasts; whilst bacteria multiply generally by fission (p. 52), and less frequently by spore formation. Under special conditions, however (p. 55), the formation of spores is also seen to occur in yeast cells.

Yeasts and moulds are closely related to one another, but are separated into two orders, the *Saccharomycetes*, and the *Hyphomycetes*, respectively. The growth in the case of yeasts is seen to be made up of round or oval cells, and there is no true mycelium; moulds are characterised by the presence of branching filaments or *hyphæ*, interlacing to form a feltwork or true *mycelium*. Hence the name *Hyphomycetes* is given to the group of moulds.

On account of their natural affinities, it is usual to study the yeasts and the moulds together. But in order to emphasise the close connection which exists between the processes of fermentation and putrefaction, and on account of the practical advantages offered by this arrangement—the organisms concerned in these processes being grouped together—it has been thought convenient to insert the lesson on the organisms of putrefaction between those dealing with the yeasts and the moulds respectively.

Yeasts, or yeast fungi, belong to the order *Saccharomycetes*, and, speaking generally, are purely saprophytic in nature (p. 37), i.e. they are unable to invade living tissues, whether animal or vegetable. This is not invariably the case, however, a notable exception being the organism of 'Thrush,' *Saccharomyces*, or *Oidium albicans* (see p. 56).

Structurally, they consist of round or oval cells, arranged singly, or in short chains. The single cells are often much larger than most of the individual bacteria such as we have already examined. Their diameter may be as much as 8 or 9·5 μ. The thin cell-wall encloses a granular protoplasm, containing one or more vacuoles, and sometimes a nucleus (best seen in old cultures, and by staining with hæmatoxylin, osmic acid, &c.).

Multiplication, as already mentioned, usually occurs by a system of budding or *gemmation*, the buds, or *daughter-cells*, remaining attached for a variable time to the mother-cell. This process is well seen in an actively fermenting saccharine solution.

In the true yeasts, *Saccharomyces cerevisiæ*, *S. ellipsoideus*, &c., under certain circumstances, three or four spores may be seen within the cell (*endospores*), whereas in the closely allied group, the true *Torulæ*, no spore formation has ever been observed at any stage of development, or under any circumstances by Hansen, whose researches, together with those of his pupils, have done so much to revolutionise the processes of modern brewing. Thus, *Torula rosea*, commonly called *pink yeast*, is no longer considered to be a true yeast. Strictly speaking, it should be included in a separate order of its own, the *Torulaceæ*, though under the microscope, apart from the question of spores, it is not to be distinguished from a yeast.

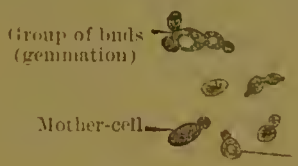

FIG. 37.—YEAST CELLS (SACCHAROMYCES CEREVISIÆ). × 900

[From Schenk's 'Bacteriology']

The conditions for spore formation in yeasts are that the cells must be young, and they should be freely supplied with air and moisture. The most favourable temperature, generally speaking, is 25° C. But the important observation was made by Hansen that at lower temperatures different yeasts require different periods for the development of spores. Thus *S. cerevisiæ* forms spores in 30 hours at temperatures ranging from 25° to 37·5° C., but it requires 10 days to do so at 11·5° C. At this lower temperature, under similar conditions otherwise, another yeast (*S. pastorianus II.*) forms spores in 77 hours. This has been turned to practical account, and thus the presence of 'wild' yeasts, as they are called, may be ascertained if contaminating yeast about to be used for brewing purposes.[1]

Appearances presented by the cultures A, B, C, D.

A. **Saccharomyces cerevisiæ** (or *beer yeast*) is a typical 'high' yeast plant (see p. 61).

Cultivations.—*Gelatine streak.*—The growth consists of raised knob-like white colonies of rounded outline. The gelatine is not liquefied, another point of difference between yeasts and moulds.

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[1] Sims Woodhead. *Bacteria and their Products.*

Under the microscope, the rounded or oval cells, already described, are seen; and also, especially if grown in a saccharine fluid, daughter-cells budding from the mother-cell, which is often vacuolated. Budding, or *gemmation*, may be replaced by endogenous spore formation, the conditions favouring this being referred to on p. 55. Their high index of refraction facilitates the detection of these endospores in unstained specimens. They have been estimated to vary from 2·5 to 6·5 μ in diameter, the yeast cells themselves being frequently 8 or 9 μ in diameter.

B. **Saccharomyces rosaceus** (*Torula rosea, the Rosa Hefe* of Germans, *pink yeast*, or *pink torula*).

Cultivations.—The raised and polished surface of the pink growth on gelatine, agar-agar, &c., closely simulates a piece of red coral.

Under the microscope, the oval cells are seen to be large, from 9 to 10 μ.

C. **Saccharomyces niger** (*Torula nigra*, or *black torula*).—Both these torulæ exist in the air, and are indistinguishable from true yeasts except by the absence of spore formation.

Cultivations on gelatine, agar-agar, &c., give rise to slightly raised shiny growth, not unlike black sealing-wax.

Under the microscope, the cells are seen to be oval, and of large size.

D. **Saccharomyces albicans** (*Oïdium albicans, thrush fungus*).—This fungus is the cause of thrush, a disease chiefly of infants, associated with the formation of somewhat circular patches of milk-white colour, occurring on the tongue and mucous membrane of the mouth. These patches, however, do not consist of a single yeast, but of a mixture of yeasts, moulds, bacteria, and epithelium (Crookshank). Sometimes it may spread down the pharynx to the œsophagus, a specimen in the possession of the writer showing the mucous membrane to be invaded by mycelial filaments. It has also been found on diphtheritic membranes and on furred tongues. An acid state of the buccal secretions seems to be necessary for active growth of the fungus, and to the excessive amount of mucus (which readily forms acid) present in infants is attributed the frequency of the disease in them compared with older children (Fagge). It also occurs in adult patients suffering from extreme exhaustion, after a prolonged illness—typhoid fever, &c., phthisis, or cancer.[1]

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[1] Fagge records frequent recoveries in spite of the unfavourable prognosis generally given in the case of adults.

Intravenous injection in rabbits and guinea-pigs has caused death in 24 to 48 hours, with the formation of long mycelial threads in the viscera (Klemperer).

Cultivations.—*Gelatine, and agar-agar, streaks.*—The growth is snow-white, and at first dry; subsequently it becomes somewhat moist and wrinkled. No liquefaction of the gelatine occurs.

Under the microscope are seen, in a young culture, rounded or oval, or cylindrical cells of varying size, but, generally speaking, considerably larger than in the case of most other yeast cells. In a medium containing sugar, the growth is purely cellular (fig. 38), and budding of daughter-cells (*gemmation*) is very obvious.

In older cultures, especially in the absence of sugar, long threads resembling mycelial filaments (fig. 39) are seen; and on this account the fungus was originally thought to be a mould. However, cultivation in saccharine and non-saccharine media suffices to prove it to be really a yeast, and like other members of this group it does not liquefy gelatine, or only slowly does so

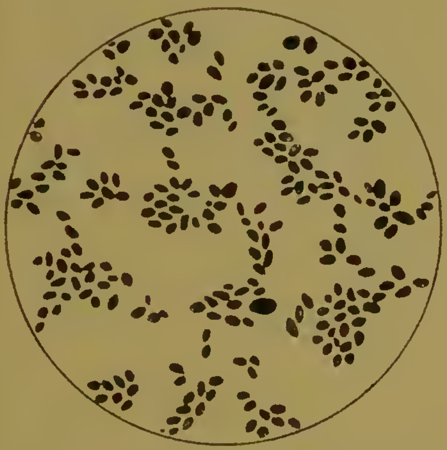

FIG. 38.—SACCHAROMYCES ALBICANS (YOUNG CULTURE). × 530

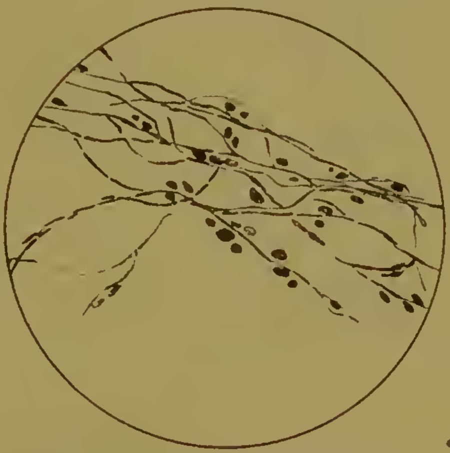

FIG. 39.—SACCHAROMYCES ALBICANS (OLD CULTURE), SHOWING MYCELIAL FILAMENTS. × 530

Saccharomyces albicans is thought to be identical with *Oïdium lactis*, the organism of sour milk.

Fermentation.—It has long been known that the addition of the yeast plant, *Saccharomyces cerevisiæ*, to a sugary solution kept at a suitable temperature gives rise to the formation of alcohol and carbonic acid. The escape of the CO_2 gas causes the solution to bubble, whence the familiar term '**fermentation**' (*fervere*, to boil). The active agent, yeast, which causes this change is called a **ferment**. For the relation between fermentation and putrefaction, see p. 67, *et seq.*

Sugar fermentation is the type of many other similar changes. We may mention a few of the best known examples:

Acetic acid fermentation, by which alcohol is converted into vinegar, the active agents being very minute bacilli, united in short chains, or curved rods (*Mycoderma aceti*, and *M. pastorianum*).

Lactic acid fermentation, in which the sugar contained in milk (lactose) is converted into lactic acid, e.g. by means of *Hueppe's lactic acid bacillus*.

Butyric acid fermentation follows upon lactic acid fermentation in milk, butter, cheese, and also in saccharine solutions. The butyric ferment is known as the *Bacillus amylobacter*, or *B. butyricus* (see also p. 60). From the spindle-shaped condition of the bacillus on the occurrence of spore formation, it is also known as *Clostridium butyricum*.

Ammoniacal decomposition of urine is also due to fermentation, in which, by means of the ferment known as the *Micrococcus ureæ*, there is a conversion of the urea into ammonium carbonate.

Other instances of fermentation, e.g. in connection with the manufacture of bread, beer, &c., will be referred to in the course of this lesson, in order to emphasise the vast commercial and economic importance of this expression of bacterial activity.

Ferments.—These are generally arranged in two classes:

I. Simple, unorganised ferments, or *enzymes*.

II. Organised ferments—yeasts, bacteria, &c.

I. **Unorganised ferments or enzymes** are conveniently classified by Halliburton[1] as follows:

- (*a*) Amylolytic, converting starch and glycogen into sugar—e.g. ptyalin, diastase, amylopsin.
- (*b*) Proteolytic, changing proteids into proteoses and peptones—e.g. pepsin and trypsin.
- (*c*) Steatolytic, splitting fats into fatty acids and glycerine—e.g. the steapsin of pancreatic juice.
- (*d*) Inversive, which convert cane-sugar, maltose, lactose into glucose—e.g. invertin of intestinal juice and of yeast cells.
- (*e*) Coagulative, converting soluble into insoluble proteids—e.g. rennet, fibrin ferment, myosin ferment.

Most of the above ferments are studied in connection with the physiology of digestion, &c., and we shall here only concern ourselves with three of them, diastase, invertin, and rennet, noting, however, that the changes produced by most ferments, organised and unorganised, consist of a hydrolytic action, or hydration, by which water is added to the starch or sugar (or cellulose –e.g. in the case of putrefactive organisms), after which a splitting-up into bodies of simpler composition occurs.

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[1] *The Essentials of Chemical Physiology*, which should be consulted for further details.

Whilst organised ferments may be killed, if the products of their vital activity—e.g. alcohol in the case of yeast—are not periodically removed, the action of unorganised ferments is merely inhibited for the time being under similar circumstances. They recover their powers when the products of their activity have been removed; so that given a suitable supply of material to work upon, and the means whereby these products may be removed continuously, unorganised ferments are capable of acting, practically, for ever.

Enzymes probably play an important rôle in the production of disease, and their importance is being more and more generally recognised. More extended investigations of these unorganised ferments, of which invertin may be taken as the type, are needed; and it is not unlikely that this is the direction along which most progress will be made in the bacteriology of the near future.[1]

Diastase is a soluble, or unorganised, ferment, which is developed in the process of *malting*. In this process, barley is steeped in water, renewed daily, for 72 to 96 hours. The water is drained off, and the barley, placed on the malting floor, is freely exposed to the air (a uniform temperature being however maintained) for 10 or 12 days, the longer period being necessary in cold weather. This encourages the barley to sprout. The developing grain, now known as *malt*, is gradually dried for three or four days, and then heated to 170° to 190° F. for several hours, to check further sprouting, and subsequently kill the young plant. The germination of the grain gives rise to the formation of the ferment **diastase**. The malt is crushed to form 'grist,' and this is thoroughly mixed with hot water in a 'mash-tun.' The mash resulting is kept at 150° F. Under these circumstances the diastase reacts upon the starch present, converting it very rapidly into dextrine and malt-sugar (maltose). The soluble products are extracted from the mash with hot water, and form what is called *wort*, from which beer is made (p. 61).

Invertin is a soluble, or unorganised, ferment, occurring in the succus entericus, and also formed during the metabolism of yeast cells. It has the power of inverting cane sugar, splitting it into dextrose and levulose.

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[1] For some of the experimental results so far obtained, the reader may be referred to *The Ferment Treatment of Cancer and Tuberculosis*, by Horace Manders, 1898.

$$\underset{\text{Cane sugar.}}{C_{12}H_{22}O_{11}} + \underset{\text{Water.}}{H_2O} = \underset{\text{Dextrose.}}{C_6H_{12}O_6} + \underset{\text{Levulose.}}{C_6H_{12}O_6}$$

This action of invertin, apart from that of the yeast cells proper, can be ascertained by using the filtrate from a mixture of yeast cells ground up with sand and water, then subjected to a pressure of 500 atmospheres, and passed through a Berkefeld filter, the filtrate[1] being free from yeast cells; or such a mixture may be heated to 60° C., or treated with ether, either process sufficing to kill the yeast cells, leaving the soluble invertin as active as ever.

Invertin has no power to convert levulose into dextrose; but the yeast cells can do so, and then both this and the dextrose originally formed by the invertin from the cane sugar can undergo alcoholic fermentation as the result of the vital activity of the yeast cells themselves.

$$\underset{\text{Dextrose.}}{C_6H_{12}O_6} = \underset{\text{Alcohol.}}{2C_2H_6O} + \underset{\text{Carbonic acid.}}{2CO_2}$$

Rennet, from which a milk-curdling enzyme or ferment is extracted, is the lining membrane of the stomach of a young calf, removed immediately after death. On the addition of salt and water, the enzyme can be dissolved out in the form of an extract. A little alcohol added to it prevents decomposition, and does not destroy its properties.

Cheese.—Rennet is used extensively in the manufacture of cheese, &c., the casein of the milk being coagulated to form a clot or *curd*, from which the liquid portion of the milk, or *whey*, is squeezed out.

Besides casein, the chief albuminoid constituent of milk, cheese contains the greater part of the cream or fatty element, and much of the mineral ash. The fermentation set up by the addition of the rennet extract does not cease when the casein is precipitated from the milk, but is carried on in the cheese during the period of its ripening or mellowing, in which the *Bacillus amylobacter* is said to play a very important part. This organism, known to be the chief organised ferment producing butyric acid fermentation (see p. 58), has been proved to exist very commonly in the stomach of ruminants, and to possess the power of coagulating milk. The casein is precipitated, and the unorganised ferment, or enzyme, associated with the bacillus then peptonises and liquefies the curd. The resulting products break down into still simpler substances, leucin, tyrosin, ammonia, &c. And almost identical changes have been shown by Duclaux to be brought about by means of an organism (similar in many respects to *B. amylobacter*) known as the *Tyrothrix* bacillus, acting on unripe cheese, the ripe cheese containing substances derived from the further breaking down of the peptonised casein (Sims Woodhead).

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[1] Buchner, to whom we owe this experiment, concludes that the 'fermentative power of the expressed juice is embodied in a soluble enzyme-like substance isolated from the living cell-plasma, undoubtedly an albuminoid,' which he terms *zymase*. For further details, see p. 140, Jörgensen's *Micro-organisms and Fermentation*. 1900.

Incidentally, it may here be mentioned that the characteristic flavour of certain well-known cheeses, Gorgonzola, Gruyère, &c., has been found by Olsen, of Norway, and others to be associated with the presence of a special organism in each case. This fact is said to have been turned to practical account, for the experimental production of any particular variety of cheese desired, by the addition of the appropriate organism to sterilised milk.

II. **Organised ferments.**—The most important fermentation produced by an organised ferment is that caused by the yeast plant, *Saccharomyces cerivisiæ,* when in contact with a sugary solution. Alcohol and carbonic acid are the chief resulting products, though small quantities of succinic acid, glycerine, and other substances are also formed.

Brewing.—In the manufacture of beer, *wort,* prepared in the way described on p. 59, is boiled with hops for two hours, the hops, besides giving an aromatic taste, exerting a preservative action. It is allowed to cool to about 16° C., and then passed from the refrigerator to the fermenting tun. Beer yeast (*S. cerevisiæ*) is then added, and fermentation at once sets in. The temperature may rise by two or three degrees.

As the yeast multiplies, bubbles of CO_2 gas rise to the surface, together with a brownish-yellow scum, which gradually increases in size. This scum, which is made up of very actively multiplying yeast cells, is removed, and may be used for fermenting a second quantity of the wort. The special yeast used in this process, from its rising to the surface, is the type of what is known as a 'high' or 'top' yeast. The temperature for this 'high' or 'top' fermentation, though only about that of the air, is considerably higher than that required in the other, or 'low' or 'bottom yeast' fermentation, viz. from 4° to 5° C.

In 'low fermentation,' well illustrated in the manufacture of the lighter ('Lager') beers, carried on so extensively in Munich, Copenhagen, &c., similar changes are produced more slowly, taking, on an average, about a fortnight, and the temperature is kept down to 4° to 5° C. Alcohol and carbonic acid are produced, as in the case of high fermentation, but the yeast falls to the bottom instead of rising as a scum to the surface.

Beer sometimes acquires a sour or bitter taste, or becomes muddy, or assumes different tints red, yellow, green, &c. These **diseased conditions** are brought about by different organisms, all of them being acid-forming.

Wine results from the fermentation of grape juice, which, in its unfermented condition, is called *must*. As in the manufacture of beer, the sugar present is split up into alcohol, carbonic acid, &c. The ferment exists in the epidermis or skin of the grape, to which it is conveyed in the form of spores by the wind. It is a yeast much like those already described, and is known as *Saccharomyces ellipsoideus I.*

Pasteur showed that **certain diseases attacking wines** are due to definite micro-organisms. Trouessart's account of these diseases and the associated ferments may be summarised as follows :[1]

(*a*) *Mouldy, or flowered, wine*, in which the white skin formed on the surface is composed of a mass of yeast cells, the *Saccharomyces mycoderma*, or *Mycoderma vini*.

(*b*) *Acidity, or sourness, of wine*, is due to the presence of the *Mycoderma aceti*, the so-called 'mother of vinegar,' a minute bacillus arranged in chains, curved rods, or figures of eight, and, as already mentioned, the common cause of acetic acid fermentation (p. 57).

(*c*) *Over-fermented wine*, when the wine is said to have 'turned.' This disease is called by the French *pousse*, and the wine *vin poussé*, or *vin bleu*, from the spurting (*pousse*) of the wine which occurs on tapping a cask, and the numerous minute bubbles seen on the surface when poured into a glass, together with the discoloration, bluish or reddish, and turbidity, characteristic of this affection. It is caused by very slender vibrios, or curved filaments, the articulations of which are barely visible.

It is a disease attacking wines of inferior quality.

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[1] For further information and illustrations, refer to Trouessart's excellent monograph on *Microbes, Ferments, and Moulds*, International Scientific Series.

(*d*) *Ropiness*, or *viscosity*, affects principally white wines, and especially champagne, the consistency of which then resembles that of white of egg. It is due to small cocci, arranged in chains, which form a dense mycelium or feltwork.

When introduced into a solution of cane-sugar, this organism first converts it into dextrose and levulose, and then the liquid becomes viscous or oily from further changes, resulting in the formation of gum and mannite (*mannitic fermentation*).

(*e*) *Bitterness* chiefly affects the better brands of red wines, and is due to bacilli in the form of distinctly articulated, curved, or bent, filaments, which are often seen in the deposit from the wine to be encrusted with colouring matter, and mixed up with crystals of tartar.

Bread.—During the manufacture of bread, the starch and sugar of the flour are acted upon by the yeast added, and, amongst other products of fermentation, alcohol and carbon dioxide are formed. One of the most important constituents of flour is *gluten*, which in the presence of moisture rapidly undergoes putrefactive changes, liberating an enzyme which starts fermentation in the starch present, thus materially assisting the action of the added yeast. The other chief function of gluten is by its 'stickiness' to bind together the moistened flour, so as to prevent the escape of the carbon dioxide gas before it has 'raised' the dough and so made the bread light and spongy. The effect of baking is to prevent further fermentative changes, by killing the ferment, and to drive off the alcohol and carbonic acid gas produced.

The agent employed to raise the flour is either ordinary *brewers' yeast*, or *German yeast*, which is compressed and partially dried brewers' yeast. *Leaven*, which is still used in some remote country districts, is merely some of the fermenting dough kept over from a previous baking. When brought into contact with fresh dough, it speedily sets up the fermentative changes already described.

Certain diseases or forms of **unsoundness** occurring in bread are due to micro-organisms. 'Bleeding bread' (see *B. prodigiosus*, p. 38) has already been mentioned. Sticky reddish-brown patches have been found associated with the presence of the potato bacillus (*B. mesentericus vulgatus*), &c. Moulds of various kinds have also been described as causes of unsound bread.

LESSON X

PUTREFACTIVE ORGANISMS—PUTREFACTION

The organisms of putrefaction.

A. **Proteus vulgaris.**

I. *Cultivations*: (i) Gelatine stab.
(ii) Gelatine plates (use 5 *p.c.* nutrient gelatine).

Examine the plates after a *few hours'* incubation, with the ⅓rd inch objective. Observe movements of individual bacilli before liquefaction has become obvious.

II. *Stain a coverslip preparation* with carbol-fuchsin.

B. **Proteus Zenkeri.**

I. *Cultivations*: (i) Gelatine stab. Note that the gelatine is, practically, not liquefied at all.
(ii) Gelatine streak.
(iii) Agar-agar plates[1] (p. 25).

II. *Stain a coverslip* with carbol-fuchsin.

III. Examine the plates daily with ⅓rd inch objective; and when the very characteristic colonies have developed make *impression preparations* (p. 31), and stain with carbol-fuchsin.

Proteus vulgaris, Proteus Zenkeri.—These two bacilli are types of a class of organisms which cause putrefaction—a term used to describe a series of chemical changes allied to those of fermentation, and referred to again in the present chapter (p. 67).

They were first isolated from putrid meat, but are found in many places where putrefaction is occurring; for instance, members of this class are found in the alimentary canal, and after death rapidly spread into all the tissues of the body. In sputum from cases of dilated bronchi, bronchiectatic cavities, and abscesses of the lung, characterised by very fœtid expectoration, long thread-like forms of putrefactive organisms are present, often in large quantities. They generally remain stained, when treated by Gram's method.

The term *Proteus* serves to indicate the great differences in length which may be met with, the organisms being sometimes short and cocci-like, or short rods, or long filaments.

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[1] Gelatine plates would do equally well, but this is a convenient and harmless bacillus to practise making agar-agar plates with, before going on to do so with more virulent organisms.

Proteus vulgaris.—Besides being seen in the places already mentioned, the *Proteus vulgaris* has been found in sewage water, and in urine in cases of cystitis (Krogins, quoted by Frankland). Subcutaneous injection into guinea-pigs and rabbits produces suppuration.

Cultivations.—*Gelatine stab.*—The gelatine is liquefied rapidly, the upper layers being of a greyish white tint, while an opaque white, flocculent growth is seen at the bottom of the tube.

Gelatine plates.—The colonies are very variable in outline, being, for the most part, more or less spindle-shaped, with numerous branches ramifying in various directions and terminating in bulbous processes. These branches are known as '*swarmers*,' or '*swarming islets*' (fig. 40), and impression preparations[1] show them to consist of numerous rod-shaped organisms, lying parallel with one another in long wavy columns, which at their free extremities coil up to form the bulbous processes. Between these elongated colonies more circular ones are seen at an early stage of growth, liquefaction occurring rapidly. They may assume a craggy, irregular, appearance, owing to central liquefaction, the growth being of a greyish white tint.

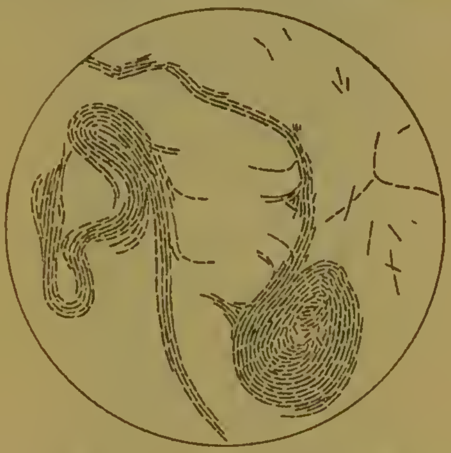

FIG. 40.—PROTEUS VULGARIS. × 530
Impression preparation from agar-agar plate three days old. Typical 'swarmers' are shown.

The growth on *agar-agar* and *potato* is moist and of a dirty greyish-white tint.

Under the microscope, rod-shaped organisms are seen, varying in length from 1 to 4 μ, and being, on an average, about 0·5 μ wide. They may be slightly curved, and very long involution forms may be present. They are very motile; spore formation is not known. It is decolourised by Gram's method.

Proteus Zenkeri is found in putrefying animal matter, and in water.

Cultivations.—*Gelatine stab.*—A fine branched growth spreads from the track of the needle. Liquefaction of the medium only slowly occurs.

Gelatine streak.—A very characteristic, finely branching, almost transparent growth is seen. The delicate branches, spreading laterally from the original streak, are curved, often with their concavity upwards, presenting an appearance which has been compared with that of a pine tree. (See fig. 41, in the upper portion of which some of the branches make a sweeping curve downwards, and have their convexity upwards.)

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[1] These should be made early the day after the plates have been poured, on account of the liquefaction of the gelatine.

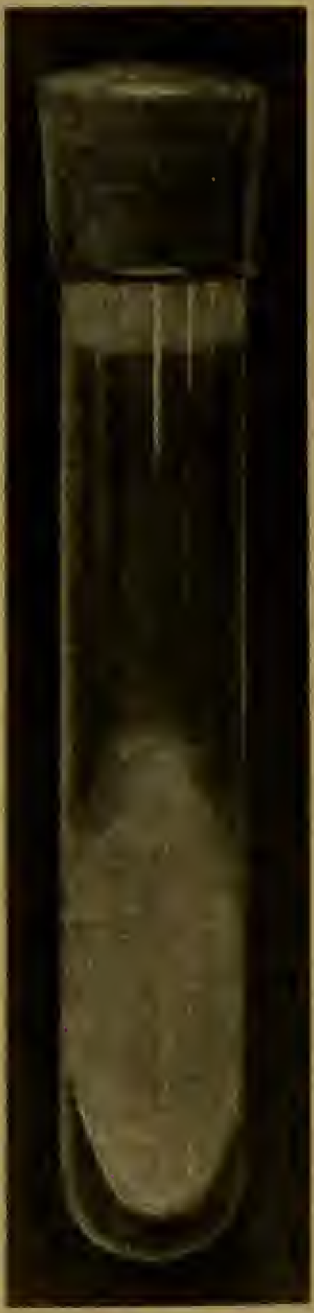

FIG. 41.—PROTEUS ZENKERI.
Gelatine streak three days old.

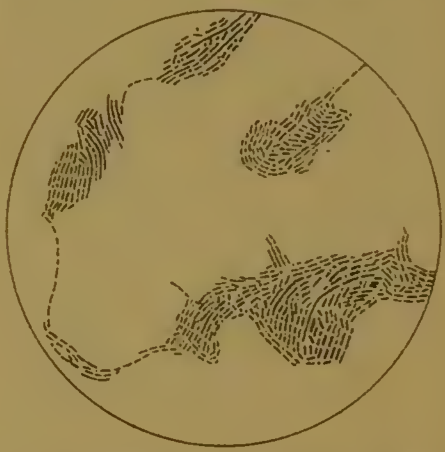

FIG. 42.—PROTEUS ZENKERI. 530
Impression preparation from agar-agar plate three days old. Typical 'swarmers' are shown

Gelatine plates.—The colonies when seen by the naked eye look like little pieces of fluff, which are often mistaken for moulds. Under a *low* power of the microscope they are seen to be of a light yellowish or greenish yellow tint, of an elongated shape, with a denser oval or circular portion, from which spring branches, passing in all directions. Many of these branches are characteristically beaded, others are distinctly spiral, and most join up with similar branches from neighbouring colonies. Between the larger ones there are discrete colonies, showing three or four branches which are coiled and may terminate in a bulbous extremity. Impression preparations (fig. 42) of the colonies show that they are made up of numerous bacilli, and their branches, which are known as '*swarmers*,' as in the case of *P. vulgaris* are seen to be made up of bacilli which are concentrically arranged in the bulbous extremity. No liquefaction of the gelatine occurs at first, and even after a considerable time it is but slightly marked. The organism is motile; no spore formation is known.

Under the microscope, every variety of shape is met with, cocci, diplococci, short rods and longer ones, threads, and involution forms. Impression preparations (fig. 42) show the typical grouping, and there is great variation in the length of the organism.

Both varieties of Proteus just mentioned exhibit a peculiar phenomenon when plates are made with 5 per cent. nutrient gelatine. At quite an early stage of incubation, a matter of a few hours only, and before liquefaction is visible, the bacilli themselves can be seen to be in active motion. As Crookshank points out, there probably is really a thin layer of liquefaction. Such motility is not observable if 10 per cent. gelatine is employed.

Proteus mirabilis.—This is another variety of the Proteus found in putrefying animal matter and water, and it appears to occur not infrequently in milk, where the long involution forms, often terminating in spindle-shaped swellings, are very characteristic. The ordinary bacilli are variable in length and shape, being sometimes round, and sometimes very thick rods with rounded ends. The organism is motile; no spores are formed.

Cultivations.—*Gelatine stab.*—A dense white layer of growth, circular in outline, forms on the surface of the medium. The liquefaction of the gelatine is not so rapid as in the case of *P. vulgaris.*

Gelatine plate.—The colonies, white to the naked eye, under the lower power of the microscope appear brownish-white, circular, or oval, and finely granular. The outline is wavy; and from it, branches pass outwards into the surrounding medium, presenting much the same appearance as is seen in the case of *P. vulgaris.* When plates are made with 5 per cent. nutrient gelatine, the peculiar movement of the bacilli, already described in the case of *P. vulgaris*, &c., can be observed under the low power of the microscope before the onset of liquefaction becomes obvious.

Putrefaction

Putrefaction is the term used to cover a series of changes similar to those of fermentation (p. 57) by which complex organic bodies are reduced to simpler chemical substances—changes which in the case of putrefaction occur after life has become extinct.

Putrefaction has therefore been defined as *putrid fermentation*, or the *fermentation of dead organic matter.* The decomposition under such circumstances is associated with the formation of characteristically offensive by-products.

Fermentation is caused by yeasts; putrefaction by bacteria, e.g. *Proteus vulgaris*, &c.

The breaking down of complex into simpler organic bodies, effected by decomposition, occurs in definite stages, each brought about by a distinct set of organisms. Thus the peculiar waxy change (*Adipocere*), seen in bodies interred in very moist burial grounds, has been ascribed to a special group of these agents.

Nitrogenous organic compounds may in this way be *de-nitrified*, or reduced to the inorganic substance ammonia and its compounds.

Under certain circumstances, in the presence of lime, potash, or other similar bases, nitrogenous organic bodies, *e.g.* ammonia and its compounds, may, however, be converted into the corresponding nitrites and nitrates of calcium, potassium, &c.

Such a change, known as **Nitrification**, is of the greatest practical importance since it is to it we owe the vast nitrate beds of Chili and Peru, &c. It, also, is due to a special group of saprophytes, the *nitrifying organisms*, to which reference will again be made.

Plants derive a large proportion of their nitrogen from the nitrates formed in this way.[1] So that whilst putrefaction is a breaking down, nitrification must be looked upon as the beginning of a building-up process.

In the superficial layers of the soil, the putrefactive bacteria thrive in oxygen, i.e. they are aërobic, whilst deeper down they are anaërobic. These deeper-lying anaërobes, though not themselves thriving in oxygen, in some cases have the faculty of seizing it from any oxygen-containing substance percolating downwards from above. This fresh supply of oxygen is then passed on to any decomposing material present, and so putrefaction, which is essentially an oxidising process, is hurried on.

Accordingly, the deeper we descend the less the nutrient material left in the soil, which, so far as organisms are concerned, is found to be sterile at the depth of about 12 feet, varying, however, with the nature of the soil, moisture, temperature, &c. Cultivated soil, possessing more organic matter than similar uncultivated ground, contains more bacteria, and these also extend to a greater depth. But below the first 18 inches the number of soil organisms falls off very rapidly (Sims Woodhead).

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[1] Reference must here be made, however, to the highly important discovery in 1886 that the nodules found on the roots of many of the Leguminosæ (peas, beans, &c.) are really due to the invasion of the latter by certain soil bacteria, special to each kind of plant. These have a remarkable capacity for fixing free nitrogen from the air. Once parasitic in the root, they give rise to these nodules (cf. the formation of oak-galls), and then multiply rapidly. The contents of the nodules become absorbed, with apparent invasion of the plant cell by the bacteria. That the associated rapid growth of the plant is not accidental has been proved experimentally by means of pure cultures.

Nitrification and the nitrifying organisms.—The process of nitrification has already been referred to, in connection with that of putrefaction (see p. 68).

The nitrifying organisms have been studied by Winogradsky,[1] and by Frankland.[2] The name *Nitro-monas* was given by the former to the chief member of the group. It has the remarkable property of thriving actively in solutions absolutely free from organic matter, and is said to be capable of deriving its necessary carbon from carbonic acid. To isolate the organisms Winogradsky selected a medium containing no organic material. Ordinary soil bacteria rapidly die out, so that only the nitrifying organisms are left behind.

Winogradsky's formula is:

Ammonium sulphate	1 gramme.
Potassium phosphate	1 ,,
Sterile distilled water	1,000 cc.

The following method is then to be adopted. Place 100 cc. of the above mixture in a series of flasks, and pour in a suspension of basic magnesium carbonate (0·5 to 1 gramme, in a little distilled water), so as to form a bed of magnesium carbonate at the bottom of the flasks. Sterilise by boiling; now inoculate one of them with a minute quantity of the soil. After four or five days a small portion is withdrawn by capillary pipette from over the surface of the layer of magnesium carbonate, and transferred to a second flask; and similarly after four or five days to a third flask, and so on. The nitrifying organisms, which eventually alone survive, remain as almost transparent films attached to the granules of magnesium carbonate at the bottom of the flask (Abbott).

Failing to grow them on the ordinary media, aërobically or anaërobically, Winogradsky has also invented a solid medium for the cultivation of these organisms, a kind of *mineral gelatine*. It is made by adding to a watery solution of silicic acid a mixture of certain salts—sulphates of ammonium and magnesium, chloride and phosphate of calcium, and carbonate of sodium. For details, the reader is referred to the very interesting account given in Abbott's 'Principles of Bacteriology,' or to the numerous papers by Winogradsky already quoted.

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[1] Winogradsky: *Annales de l'Institut Pasteur*, for 1890, 1891 (numerous papers).

[2] Refer to Frankland's *Our Secret Friends and Foes*.

Under the microscope, the Nitromonas is seen as short, oval, often almost spherical, non-motile cells, arranged singly, or in zooglœa masses. Multiplication occurs by fission; spore formation is not known.

The Nitromonas is found in the superficial layers of the soil everywhere, but certain places seem particularly favourable to its growth. Thus in Peru and Chili there are immense beds of nitrates produced from the ammoniacal guano, or excrement of sea-fowls, as the result of the activity of these nitrifying organisms.

Similar processes of decomposition and nitrification occur in sewage allowed to filter through the soil in a sewage farm. Chiefly on account of the presence of these nitrifying organisms Dr. G. V. Poore has long and ably advocated (in his 'Rural Hygiene,' and elsewhere) [1] the disposal of fæces and urine, as well as of dead bodies, by burial beneath the superficial layers of cultivated soil.

LESSON XI

MOULDS

A. **Penicillium glaucum** B. **Aspergillus niger** C. **Mucor mucedo**

I. *Make cultivations* of A, B, and C on

(i) Gelatine stab. (ii) Agar-agar streak.

(iii) Potato (keep at the temperature of the room).

II. *Make coverslip preparations.* These require special methods.

A. **Penicillium glaucum** contains so much fatty material in its mycelium that the best way to mount it is as follows:

By means of a loop or hook of platinum wire some of the culture is transferred to a clean watch-glass, and covered by another till required for use. From this a small fragment is taken by means of the platinum wire or fine-pointed forceps, and placed in a watch-glass containing two or three drops of liq. ammoniæ in strong methylated spirit. The fat is extracted to some extent after some five to ten minutes' soaking, and the fragment is then gently transferred to a drop of glycerine on a slide, and covered with a coverslip.

To stain the preparation, Löffler's methylene blue may be allowed to diffuse beneath the coverslip, over-night.

If glycerine is used, the coverslip should be sealed down by painting the edges with freshly melted paraffin. Glycerine jelly may be conveniently substituted for glycerine, and it is best applied after melting it thoroughly in some hot water. When quite liquid, a small quantity is placed on the clean slide, the mycelium (after maceration in the ammoniated spirit, in the way described) is transferred with fine forceps to the centre of the jelly, and the coverslip then applied. The glycerine jelly sets fairly firmly after some days, but it is safer to seal the edges of the coverslip by painting them over with melted paraffin. If desired, a little Löffler's blue may be dropped on to the jelly whilst still liquid, so as to stain the preparation.

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[1] A discussion of this question is to be found in the *Transactions of the Royal Medico-Chirurgical Society* for 1897.

B. and C. For **Aspergillus niger**, **Mucor mucedo**, and similar moulds, proceed as follows:

Some of the growth is removed from the culture-tube by means of a platinum loop or hook. It may be kept between two watch-glasses till required. With fine forceps a few filaments are taken up and transferred to a drop of melted glycerine jelly placed in the centre of a slide. Apply the coverslip, and seal the edges with melted paraffin. In the case of the *Mucors*, Löffler's blue may be dropped into the melted glycerine jelly. If glycerine be used instead of glycerine jelly, the stain may be allowed to diffuse under the coverslip over-night.

N.B. In the case of *Aspergillus*, it is essential that quite a young culture be used, whilst it is still white, before spore formation has made much progress. Otherwise the structure of the conidiophores will be completely obscured by black spore-masses.

Moulds

Penicillium glaucum.—This is the commonest of all moulds, and must be familiar to every one as a fluffy white growth, which becomes either bluish or greenish, and may be covered with drops of a dew-like fluid. The change from white to blue or green indicates the formation of spores.

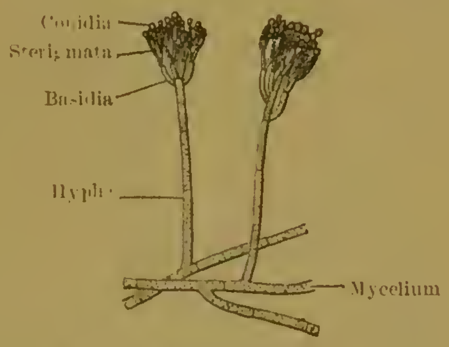

FIG. 43.—PENICILLIUM GLAUCUM. × 400
(Schenk, after Baumgarten.)

Cultures.—*Gelatine stab.* A thick scum is formed on the surface of the medium, which is liquefied in a short time. The surface of the scum is at first white, fine hairs proceeding horizontally, and, also, in a radiating fashion from the centre of the colony downwards into the medium. The colour changes to a blue, or a bluish-green, in the course of a day or two. On *agar-agar* and *potato* similar changes are seen, except for the liquefaction.

Under the microscope, a mycelium is seen, from which branches or *hyphæ* extend. These hyphæ split up into a number of finger-like processes, or *basidia*, consisting of cylindrical cells, splitting up into branches, called *sterigmata*, which in turn break up into rounded or oval, spore-like bodies, or *conidia*, which are of a bluish or greenish colour. At first these conidia are closely connected, but eventually separate from one another, and so become scattered far and wide.

Aspergillus niger.—This organism occurs on bread, and several species, *A. fumigatus*, *A. flavus*, &c., have been found saprophytic, and sometimes definitely parasitic and pathogenic,[1] in man—e.g. the external and middle ear, lungs, kidneys, &c.

Cultures.—*Gelatine stab.*—A white, dense, felt-like growth appears at first with liquefaction of the subjacent medium. In a day or so minute black points are visible, indicating the formation of spores. The white growth then becomes almost entirely black. This appearance is especially well marked on potato, the white colour of this medium appearing in sharp contrast to the dead-black growth.

Under the microscope, the mycelium is seen to be jointed, the club-shaped head or extremity of one joint fitting into the concave end of the next. Branches are given off at fairly regular intervals; the appearance generally is very like that of bamboo.

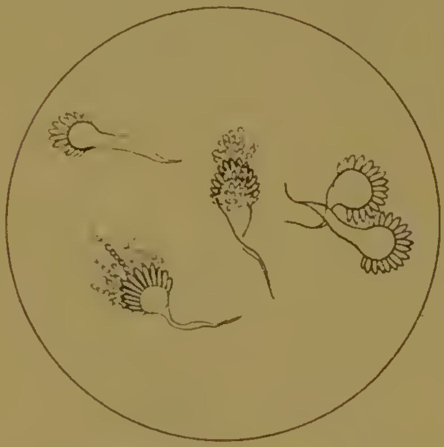

FIG. 44.—ASPERGILLUS NIGER. × 420
(No. 4 ocular: $\frac{1}{6}$-inch objective.)

From the mycelium, *fruit hyphæ* proceed upwards into the air. These consist of elongated cells, the free ends of which are swollen up to form a club-shaped enlargement (fig. 44). The conidiophores on the surface of these club-shaped elongated cells appear as flask-shaped *sterigmata*, from which proceed spores or *conidia* which, like those of penicilium, are at first adherent to one another, but soon break loose, and are scattered everywhere. The flask-shaped cells and the first row of conidia are not unlike a row of skittles; but to obtain this appearance it is necessary to make a preparation while the growth is still white, before spore formation has gone too far, otherwise the appearance is obscured by a multitude of black spores.

Mucor mucedo is the common white mould, and is found on rotten fruit, potatoes, nuts, mouldy bread, the excrement of horses and other animals, &c. *Sleepiness*, the term applied to the first stage of rotting, so characteristically seen in pears, has been attributed to the presence of this mould by Davaine.

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[1] Such conditions are indicated by the terms '**Aspergillar mycosis**,' '**Pneumomycosis**,' &c. Consult the article on '**Mycoses**,' by Professor J. Rose Bradford, F.R.S., in *Allchin's Manual of Medicine*, vol. i., 1900.

Cultivations.—*Gelatine stab.*—On the surface of the medium a dense mycelial growth is seen, with branches passing upwards and downwards: the down growths, known as *sub-aërial hyphæ*, extend into the gelatine, which soon liquefies; the others, passing upwards into the air, are called *aërial hyphæ*, which are well compared with 'spun glass,' from their glistening appearance. The ovoid upper[1] extremity, or *columella*, of each hypha is to some extent screened from view by a terminal rounded knob, or *sporangium*, in which ovoid spores develop under favourable circumstances in the course of a few hours. On rotten fruit, for instance, the spores of this mould germinate in five or six hours by introducing their hyphæ through the epidermis (Trouessart).

There are two other common varieties of mucor, *M. corymbifer* and *M. rhizopodiformis*; both occur on bread, the former as a dense, snow-white growth, sometimes seen in the external auditory meatus. Both are pathogenic when intravenously injected into rabbits. *M. aspergillus* has dark-brown spores, whilst several other varieties of mucor have black sporangia, e.g. *M. stolonifer*, *M. phycomyces*, whilst *M. spinosus* has chocolate sporangia, and short processes, or spines, on the columella (Crookshank).

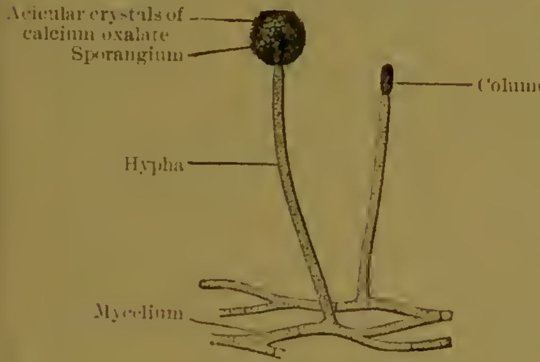

FIG. 45.—MUCOR MUCEDO.
(Schenk, after Baumgarten.)

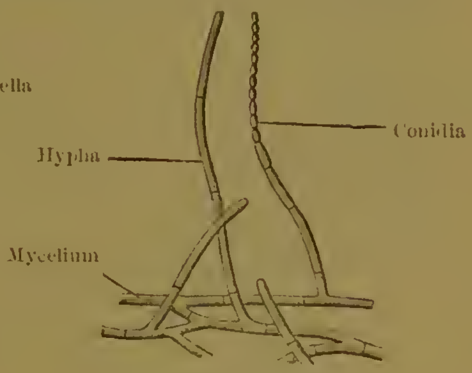

FIG. 46.—OÏDIUM LACTIS.
(After Baumgarten.)

Oïdium lactis, one of the common organisms producing sour milk, a lactic acid fermentation, is frequently placed in the same class of hypho-mycetes, but it does not liquefy gelatine. There is a mycelium formed of branching filaments, from which arise true hyphæ, which in turn end in a chain of spores, or conidia.

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[1] The columella appears to form a prominence projecting into the sporangium. This is characteristic of the mucors and is of diagnostic value.

Microsporon furfur, the organism of *Pityriasis*, or *Tinea, versicolor*, (formerly called *Chloasma*), a disease in which there are patches of light-brown, or fawn-coloured pigmentation of the skin over the chest, abdomen, thighs, &c., is probably identical with *Oïdium lactis*, which, as mentioned on p. 57, is thought to be identical, also, with *saccharomyces (or oïdium) albicans*.

Oïdium albicans, the organism producing the white patches on the tongue of infants known as 'thrush,' was formerly included amongst the moulds; but there are reasons for believing it to be a yeast, so that it is now more generally known as *Saccharomyces albicans* (see p. 57).

LESSON XII[1]

The moulds of ringworm and favus

Culture media.—All the ordinary media may be used for these fungi: nutrient broth, gelatine, glycerine agar, maltose agar, potato, &c. Wort gelatine and wort agar (p. 8) are excellent; but, in the case of ringworm, it is better to use the formula given below.

For the organism of favus, wort agar, or glycerine agar, may be employed. Saccharine bodies, whilst favouring the growth of ringworm organisms, do not assist the development of that of favus.[2]

For the organism of ringworm, the medium preferred by Sabouraud and extensively used by him for the differentiation of the varieties of ringworm fungi, is a maltose agar, generally known in England as

French proof agar (Sabouraud's *milieu d'épreuve*).—It is made, just like sugar agar, from the following formula:

	Grammes.
Distilled water	100
Peptone (Chassaing's)	0·7
Maltose	3·8
Agar-agar	1·3

English proof agar is the name given by Blaxall to a medium made according to the above formula, except that the peptone was of German origin (Witte's), and the pure maltose was obtained from English chemists. The cultures obtained were excellent and easily differentiated, but they presented appearances quite unlike those described by Sabouraud, these being, however, at once obtained on working with the same maltose as Sabouraud had used.

It is, therefore, absolutely necessary in comparing the various forms of ringworm fungi always to use a medium made strictly according to a specified formula, and exposed to the same physical conditions—e.g. during its manufacture, &c.; and for this purpose, too, young cultures of the trichophyta should be used for comparison and for making sub-cultures, especially such as have been seen to spring from a single spore, according to Blaxall's plate method (p. 75). Under these circumstances the trichophyta invariably breed true. The best temperature is 30° C.

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[1] This lesson may be judiciously omitted by the beginner, though the staining of hairs and scales affected with ringworm and favus, according to the directions given, should be practised, if there is time.

[2] Refer to pp. 94-95.

Method of inoculating the diseased hair, crust, &c.—The method generally adopted is to transfer the hair, &c., to a clean slide previously sterilised by flaming. The unaffected part of the hair is cut away with a sterile knife, and the remainder subdivided into small portions, which are transferred to a tube containing a suitable medium by means of the platinum needle. The point of the needle should, however, have been previously rendered adhesive by plunging it whilst still warm into the medium, or by moistening it in the condensation water.

This is sufficient for the microsporon hairs, which, as Sabouraud and Blaxall state, yield a pure culture of the parasite when placed on a suitable medium inimical to saprophytic bacteria. The hairs and scales of the trichophyta are, however, frequently infested with bacteria, yeasts, sarcinæ, and moulds. The difficulty arising from growth of the latter can generally be obviated by teasing out the material in sterilised distilled water, separating the diseased hairs by means of a lens, and again washing them in distilled water. This is especially indicated for scales from *Tinea circinata*, also in beard cases, and in the crust and matted hairs obtained from animal lesions (Blaxall).

Should the growth obtained be not a pure ringworm fungus, it should be removed and teased well in sterile distilled water and re-inoculated.

Both in the case of favus and ringworm, if typical colonies appear to be sprouting in any of the tubes amidst contaminating organisms, subcultures are made on fresh tubes, so as to isolate pure growths.

Plate cultivations.—These may be made in the case of ringworm from a tube culture of either the microsporon or the trichophyta, and are especially useful for the trichophyta, where otherwise only an approximately pure culture can be obtained, for the reasons already indicated. The method of plating devised by Blaxall is founded on the fact, noted by Sabouraud, that the trichophyta generally (and also the microspora when quite white and downy), as soon as they have pushed up their aërial hyphæ, are exceedingly prone to drop their spores and thus cause little satellite colonies around them.

Blaxall's method.—A tube culture of a *trichophyton* which had thrown up its aërial hyphæ is taken, and after removal of the wool plug, the mouth is well flamed. The tube is then held inverted over a petri-dish containing maltose agar solidified. A sharp tap or two is given to the tube, sufficient to cause the spores to drop, and the plate is re-covered.

Under the low powers of the microscope the spores can be sought out and watched, the development from a single spore followed, and subcultures made from isolated colonies.

By this method the trichophyton may be separated from its *commensals* (see p. 92), or rather their reappearance is considerably, if not entirely, checked. As soon as there is any sign of growth the plates are sealed with melted paraffin.

Impression preparations can be made from these plates, in the usual way, in order to demonstrate the aërial hyphæ in their natural arrangement. Blaxall recommends Kühne's carbol-methylene-blue as a stain. After about five minutes in this, well wash with tap and distilled waters; dry and mount.

Examination of the submerged hyphæ.—Blaxall's method is to place a thin section of the agar containing the submerged hyphæ between two coverslips. These are pressed tightly together and can be examined at once fresh; or, by holding the two cover-glasses clipped tightly together in boiling water, most of the agar can be removed and the specimen stained.

In this way Blaxall 'has been able to confirm the appearances seen in a hanging-drop specimen, and to observe that the appearances of the submerged hyphæ of the microspora correspond exactly to the description given by Sabouraud as to the fructification of that species.'

Hanging-drop specimens are best made, according to the same writer, by using as the medium beer-wort, or a bouillon containing maltose, or, as recommended by Bodin, mannite. After inoculation the coverslip is hermetically sealed to the cell of the hollow-ground slide with melted paraffin. Cultures can be watched for over a month.

Methods of demonstrating fungi in the hair, in cases of favus or tinea tonsurans.—The hair should be carefully extracted from the scalp, &c., by traction in the axis of its growth, so as to obtain the soft bulb intact if possible.

I. **Rapid clinical method.** 1. Place the hair in a drop of liquor potassæ, 5 to 10 per cent. (the B.P. solution, 6·8 per cent., is very convenient), on a clean slide.

2. Lay the coverslip on gently, but do not press. Examine the hair during the process of clearing. Do not warm or disturb the hair.

[Adamson says that previously soaking the hair in ether is useful, especially in the presence of scales or fat, e.g. pomade, as it prevents the softening action of the potash. But as a rule it is not necessary.

3. After fifteen to twenty minutes allow a little glycerine to diffuse under the coverslip, which may then be luted down by painting its edges with melted paraffin.

II. **Adamson's method**[1] unites the foregoing with a staining process, the Gram-Weigert method (p. 99), a happy combination, which I find gives most excellent results.

The method of procedure is as follows:

A. '*Clearing' process.*—1. Place the hair or scales on a slide, and cover with a drop of potash solution, 5 to 10 per cent. (or B.P. solution, 6·8 per cent.). The cover-glass is gently applied.

2. After ten to thirty minutes (for details, see p. 78), gently wash the specimen in a 15 per cent. solution of alcohol in water, by carefully diffusing a few drops under the coverslip. The alcohol mixing with the potash solution hardens the specimen sufficiently to prevent its destruction during the removal of the cover-glass. In the case of scales these will remain adherent, either to the coverslip or the slide, while a hair usually floats, and must be left behind by the removal of alcohol with blotting-paper. Any excess of potash is then washed off with more 15 per cent. alcohol.

3. The slide, or cover-glass, bearing the specimen is dried over the flame, and, in the case of scales, fixed by passing through the flame three times.

The preparation is now ready for staining, Gram-Weigert's method (modified as to times, p. 78) being used for demonstrating the fungus, whilst the hair itself may be counterstained by adding eosin or picric acid to the anilin oil, as follows:

B. *Staining process.*—4. Stain in anilin-water gentian-violet, for fifteen to sixty minutes (p. 78).

5. Pour off the stain, and, without washing, pour on Gram's iodine solution. Pour this away after one to five minutes.

6. Decolorise in anilin oil, for two to three hours or longer; renew the anilin oil, occasionally. Examine continually with the ⅓rd inch objective.

7. Remove the anilin oil by blotting-paper, and mount in Canada balsam.

I add to Adamson's account the following details as to the mode of counterstaining the hair, which is simple and very effective.

When the spores and mycelium are clearly seen, and the hair is fairly free from gentian-violet, pour on a drop or two of concentrated alcoholic solution of eosin. After one minute wash away excess of eosin with a little more anilin oil. Then absorb excess of oil with blotting-paper, and finally remove all traces of oil with xylol, to prevent decolorisation of the hair going on.

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[1] 'A note on the Permanent Staining of Ringworm Fungus,' by Dr. H. G. Adamson, *Brit. Journ. Dermatology*, Dec. 1895, pp. 373–377.

Absorb excess of xylol. Mount in xylol solution of Canada balsam.

The fungus is stained violet, and the hair or scale pink.

As regards the 'clearing' stage, Adamson says: 'The time of exposure to the potash solution varies according to the rate at which clearing, as manifest under the microscope, takes place; or according to the particular appearance it is wished to record, the time being least when very superficial parts of the hair or fungus are to be seen.

'*To show the fringe of mycelium at the neck of the soft bulb* in either of the varieties of ringworm, the potash is only used for a few minutes. So for *ringworm of the beard,* where the external 'sheath' is to be demonstrated.

'*When demonstrating the fungus within the hair* in the endothrix forms, or in the thick hairs from the beard, the specimens must soak for a longer period.'

'*Again, for staining the elements of the fungus simply,* without regard to their relations to the hair, a longer period is required, and the specimen may also be flattened out by pressure of the coverslip.

'*As regards the staining and decolorising,*' the author continues, 'the length of time will depend on the exposure to potash and the consequent softening produced.

'*Where the potash is used for a long time,* a few minutes only in the stain, and fifteen to thirty minutes in the aniline oil, will be sufficient.

'*Where the potash has been used only for a few minutes,* the staining and decolorising both require a longer time, viz. staining half to one hour or longer, and decolorising for two to three hours or longer.'

It may be mentioned that, without troubling about minutiæ, the present writer has obtained very satisfactory results with Adamson's method (see figs. 47, and 56), using the following **average times**:

Place in potash for twenty minutes.

Wash in 15 per cent. alcohol.

Stain in anilin-gentian-violet, for thirty minutes.

Pour on Gram's iodine solution, three minutes.

Decolorise in anilin oil, thirty minutes.

Counterstain in eosin one minute (see p. 77).

Ringworm[1]

The parasitic nature of ringworm was first accurately described by Gruby, in a series of memoirs published in the *Comptes rendus*, Paris, between the years 1841–1844. The existence of varieties of fungi mentioned by Gruby has, during the present decade, been confirmed and our knowledge of them vastly extended by the brilliant work of Sabouraud, also of Paris, and his observations have been further confirmed, or qualified, by the very careful investigations of Adamson, and of Colcott Fox and Blaxall amongst English writers.

True ringworm is caused by moulds of two kinds, the first being the **Microsporon Audouïni**, so called by Gruby, the other belonging to the family of the **Trichophyta.** The Trichophyton fungi are distinguished as **T. endothrix** or **T. ectothrix**, according as they are seen microscopically to occupy chiefly the interior or the exterior of the hair, respectively.

Occasionally, owing to the absence of the characteristic favus cups, and the presence of circinate lesions, or even typical kerion, favus may so closely simulate ringworm as to be indistinguishable from the latter clinically. When cultivated, however, the fungus gives rise to typical favus growths. Such a condition has been called by Sabouraud '**Favus with trichophytoid lesions.**'

The microsporon and trichophyton fungi are quite distinct from one another, morphologically, culturally, and, according to Sabouraud, botanically. Fox and Blaxall, however, maintain that they must all be regarded as members of the same family, basing their argument on the similarity in the plan of the aërial fructification seen in the *cultures* of all true ringworm fungi, viz. a central rod bearing terminally and laterally small spores,[2] attached by a short pedicle.

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[1] The literature of the subject, owing to the large number of investigators, is very great. The account here given is based chiefly upon the work of Sabouraud, Adamson, and Colcott Fox and Blaxall. The two last mentioned have written an admirable series of papers in the *British Journal of Dermatology* for 1895 and 1896, and a short account will be found in the Trans. Path. Soc. Lond. for 1897. Having most courteously placed at my disposal six of the cultures (figs. 50–55), and two hairs affected with trichophyton (figs. 48 and 49), mentioned in their original papers, I have availed myself of the descriptions therein given. From the recent nature of the investigations it has been necessary often to quote freely therefrom. The advanced student will find them invaluable for the purposes of reference, owing to the vast amount of detail given.

[2] As the writers quoted point out, the aspect of the aërial fructifications of these cultures has nothing to do, and must not be confounded with, the 'spores' described in the hair lesions.

The fungi producing ringworm, and the ringworm-like lesions yielding faviform cultures, are conveniently arranged into three groups.

I. **The Microsporon Audouïni,** a fungus the mycelial filaments of which, according to Sabouraud,[1] occupy the interior of the hair, whilst the small spores form a characteristic greyish-white sheath outside the hair, spreading along its shaft for a distance of 3 mm. (about $\frac{1}{8}$ inch) from the mouth of the hair follicle. It is responsible for the majority of cases of scalp ringworm of children in England.

II. **The Trichophyta,** also known as **Trichophyta megalospora,** from the large size of the spores ordinarily seen, are further subdivided into (*a*) **endothrix** and (*b*) **ectothrix** varieties, according as the fungus lies chiefly inside or outside the hair; (*c*) a third variety has been distinguished by the name **endo-ectothrix,** the fungus lying partly inside and partly outside the hair. From its clinical behaviour and cultural characters it must be regarded as being closely allied to, if not identical with (*b*). *Trichophyton megalosporon endothrix* forms no white parasitic sheath external to the hair; whereas the *ectothrix* variety, lying almost entirely outside the hair, does form a parasitic sheath, much as in the case of the microsporon.

III. **Fungi causing ringworm-like lesions but favus-like cultures** (i.e. '*favus with trichophytoid lesions,*' see p. 95).

Reserving to the end of the chapter the further consideration of group III., we may conveniently compare the parasites of true ringworm under the following headings: Source, Mode of invasion of hair, Frequency, Clinical and Microscopical appearances, and Culturally.

Source of the ringworm fungi.—(i) The *microsporon Audouïni* is thought to be of human origin, but a species of microsporon is known in the horse and also in the cat; and such microspora of animal origin appear to produce circinate lesions of the smooth, or glabrous, skin more commonly than is the case with *M. Audouïni*.

(ii) *The trichophyton endothrix* is of exclusively human origin.

(iii) The *trichophyton ecto-* and *endo-ectothrix* appear to be of animal origin.

Mode of invasion of the hair.—(i) *The microsporon,* according to Sabouraud, attacks the hair near the mouth of the follicle, eroding its surface and then spreading from above down. The scalp epidermis is only attacked subsequently, according to Sabouraud; but Fox and Blaxall think that the hair is attacked secondarily to the skin of the scalp.

(ii) and (iii) *The trichophyta,* descending into the follicle, attack the hair near its root, the fungus spreading upwards. The remarkable preservation of the cuticle points to a method of attack different from that in the case of the microsporon (Fox and Blaxall).

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[1] See, however, p. 82, *et seq.*

In scalp ringworms of *trichophytic* origin (far less commonly met with) there is a preliminary and temporary circinate lesion of the epidermis around, before the hair is invaded by the fungus, and there are frequently circinate lesions of the smooth skin elsewhere.

But, according to Fox and Blaxall, 'no rule can be laid down as to the exact spot where the microsporon first attacks the hair. It is always in the follicular portion, and often towards the root end, but sometimes in one place, sometimes in another, and on occasion in several.' (For details as to the mode of this invasion, see p. 83.)

Frequency.—The proportion of small-spored to large-spored fungi in scalp ringworm varies considerably. In Paris, it is as 12 to 8. In England, the small-spored variety is largely in excess. Thus out of 178 cases, Adamson [1] observed only 7 instances of large-spored forms, a proportion of small-spored to large as 25 to 1.

Clinically.—(i) The *microsporon Audouïni* is responsible in England for the majority of the cases of scalp ringworm in children, various estimates showing it to be present in from 80 to 95 per cent. of all cases. In Paris it causes a little more than half, and less than two-thirds of the total number of cases. It appears not to exist in Italy, Germany, or Hungary.

Fairly long stumps of broken hairs, each surrounded by a white sheath, are found throughout the affected areas. In the adult it is but rarely met with in the scalp, and it is uncommon for it to attack the smooth skin.

(ii) The *trichophyton endothrix*, common in Paris, less common in England, frequently affects the smooth skin, producing, however, less than half of the total number of circinate lesions. When associated with scalp lesions, very short, swollen, and dark stumps, having no parasitic sheath externally, are characteristic. Two types of scalp lesion are described by Sabouraud; the one, designated by him under the name '*La tondante peladoïde*,' from its close resemblance to *Alopecia areata*, has not been seen in London. The second type, also rare in London, is characterised by the presence of numerous small scurfy patches, 'the size of the finger-nail,' or smaller, or of single stumps scattered over the scalp, and for which careful search may have to be made. Sometimes, however, large patches may be present.

(iii) The *trichophyton ecto-* and *endo-ectothrix* fungi are responsible for only a small minority of scalp ringworms, but for more than half the total number of circinate lesions of the smooth skin (*Tinea circinata*).

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[1] *Brit. Journ. Dermatology*, July 1895.

These fungi are also the cause of all beard ringworms (*T. sycosis*), and ringworm of the nails, a very rare disease in England, in which the nails become of a dirty yellowish colour, thickened at the edge, and very brittle, so as to split readily.

Kerion, a condition of ringworm characterised by a spongy swelling of the scalp from suppurative folliculitis, is also invariably due to these fungi; but before making the diagnosis of ectothrix one must exclude the other common sources of suppuration in the scalp, the ordinary pyogenic organisms, pediculi, &c. (Fox and Blaxall).

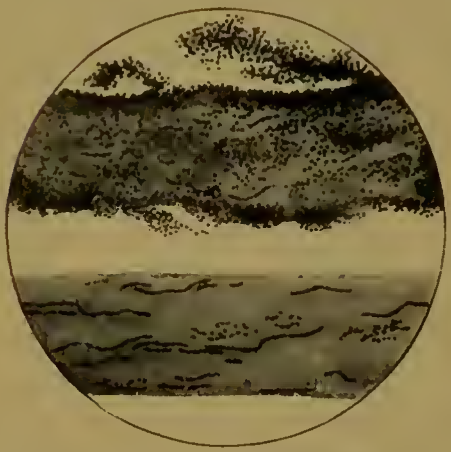

FIG. 47.—MICROSPORON AUDOUÏNI IN A HAIR, FROM A CASE OF SCALP RINGWORM. × 240

Stained by Adamson's method. In the upper figure the surface has been focussed so as to show the mosaic of spores forming a sheath outside the hair. In the lower figure, the same hair has been focussed to show the mycelium. Notice that the cuticle of the hair has been stripped off.

Microscopically.—(i) *The microsporon Audouïni hair* (fig. 47), when prepared by either of the methods already described, is seen to be enclosed in a sheath composed entirely of small spores—the majority being 2 to 3 μ in diameter—fitting closely together so as to form a mosaic; but there are no definite filaments or chains of 'mycelial spores,' as in the case of *trichophyton ectothrix*.

'In a carefully extracted hair,' Adamson says,[1] 'the whole root-stem is seen surrounded by the sheath of spores, extending from a short distance above the intrafollicular portion to the junction of the root-stem with the soft root-bulb. The hair itself is free from spores.

'On the bulb are strings of spores, radiating from the papillary centre, but these may be only picked up by the expanded soft bulb as it passes through the follicle, upon whose wall some portion of the spore sheath may still remain.

'There is no mycelium on, or in, the bulb; but at the junction of the bulb with the root-stem there is a *fringe of mycelium* surrounding the hair and projecting below the lower margin of the sheath of spores around the root-stem.

'By careful focussing it can be seen that the mycelium extends upwards beneath the sheath of spores to form an internal sheath between it and the hair. The mycelium is most abundant at the lower part of the root-stem, becoming more broken and scanty as the aërial portion of the hair is reached. The spore-sheath passes a little way on to the aërial stem, and above this are scattered spores and broken rods of mycelium. In the aërial stem there usually exist one or more cross-fractures of the hair. At this stage the cuticle of the hair is destroyed throughout its whole length, and the mycelium appears imbedded in the surface of the hair.'

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[1] *Brit. Journal Dermat.* July 1895.

Besides these small spores, however, there may be seen, here and there, on the outside of the hair when in an early stage of infection, larger masses or segments, the so-called *giant spores*.

'They are usually seen as thinly scattered groups, or isolated chains, on the surface of the shaft, at the junction of the follicular with the aërial portion of the hair, and sometimes as chains of large-jointed mycelium nearer the root. The elements are quite different in appearance from those of the main fungus. They are somewhat irregular in outline, and refract light only slightly, so that they present a faint ghost-like appearance.

'Whilst in many of the hairs in the early stages of infection no spores are visible, in others there is a more or less imperfect sheath of small spores, and in these the larger elements just described are always present. When traced downwards they are seen to pass through all the intermediate stages into the typical small spores of the sheath. It would thus appear possible that the larger spores are the elements from which the spore-sheath takes its origin.' (Adamson.)

Jointed mycelial threads, according to Sabouraud, however, occupy the interior of the hair, and appear to branch dichotomously at long intervals. The branches end in fine filaments, which, escaping through the hair, terminate by bearing spores. These, being packed closely side by side, present the appearance of a mosaic, and form the greyish-white sheath which is visible to the naked eye.

Sabouraud considers that the spore thus formed on the outside of the hair is an ectospore, as distinguished from a mycelial endospore (such as is seen in the trichophyta, or in favus), which is only a portion of altered mycelium. He believes that the microsporon is the only one of the ringworm fungi capable of passing through a complete developmental cycle, i.e. by the production of ectospores, whilst parasitic in the human subject. Fox and Blaxall, in opposing this view, refer to the characteristic mode of invasion of the hair by the large 'giant spores.'

The exact mode of invasion of the hair is best studied in specimens obtained from the early scaly patches of the scalp before the hairs become broken. Adamson's account is as follows :[1]

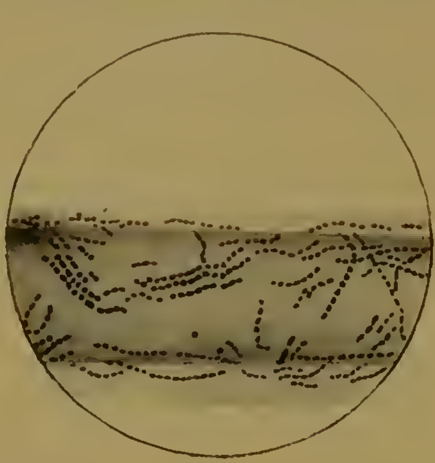

Fig. 48.—TRICHOPHYTON ENDOTHRIX IN A HAIR.[2] × 530

The chains of mycelial spores are mostly confined to the interior of the hair, of which cuticle is seen to be remarkably well preserved. N.B.—In this and the succeeding figure it has been necessary, for the sake of clearness, only to represent a few of the chain formations actually present.

'In many of the hairs abstracted from such an early patch nothing abnormal can be seen ; but, here and there, a hair is found with its cuticular covering intact but invaded by mycelium,[3] which can be seen piercing it from the outside, at the upper part of the follicular shaft, and passing downwards as long wavy threads towards the root. It lies immediately beneath the cuticle, and terminates as a *fringe* just at the neck of the soft bulb.' The cuticle is then progressively stripped off in a very characteristic manner, in marked distinction to what happens in the case of the trichophyton, in which the cuticle is well preserved. This, according to Fox and Blaxall, shows that the fungi attack the hair in a different way in the two cases.

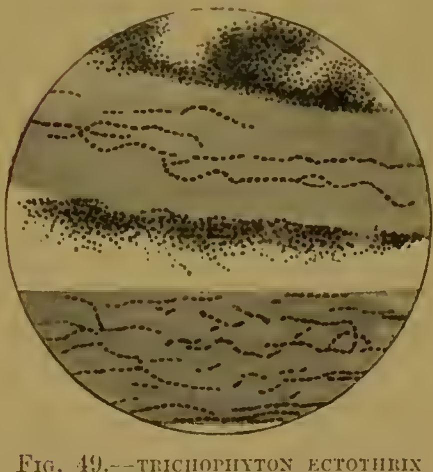

Fig. 49.—TRICHOPHYTON ECTOTHRIX IN A HAIR.[2] × 530

From a case of kerion. In the upper figure the hair has been focussed so as to be seen in profile. The massed chains of mycelial endospores lying on the exterior of the hair are well seen. In the lower figure, from the same specimen, the surface of the hair is focussed, but only a few of the very numerous chains of mycelial endospores are shown.

(ii) *The trichophyton endothrix hair* (fig. 48) is seen to be devoid of a parasitic sheath, but to have retained its cuticle to a remarkable extent. The root is first attacked, and the parasite grows upwards. Within the hair, and occupying it more or less completely, are mycelial filaments, broken up to form chains of double-contoured spores —*mycelial spores*, or *endo-spores*— which as a rule are larger than in the case of the microsporon. Only exceptionally is any of the fungus seen outside the hair (p. 85).

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[1] *Loc. cit.*

[2] See 1st footnote, p. 79.

[3] This mycelium is really derived from 'giant spores' (p. 83) lying on the shaft of the hair.

This chain formation is often less distinct in the peladoid type (p. 81) of ringworm, the rounded or irregularly shaped spores being massed together to look 'like a bag of nuts' (Blaxall). In the second endothrix type (mentioned on p. 81), the chain formation is obvious, and the spores are mostly quadrangular in shape.

(iii) *The trichophyton ectothrix hair* (fig. 49), e.g. from a case of suppurating ringworm (*kerion*) or in beard ringworm (*sycosis*), has a dense white or greyish parasitic sheath, chiefly confined to the intrafollicular portion. The fungus may be practically limited to this region, where it is seen to form an ensheathing mass of mycelial threads between the hair and the wall of the follicle. But, as Fox and Blaxall point out, the hair itself is probably always implicated to some extent, and the wall of the follicle may be attacked. But a more extensive invasion of the hair by the parasite may occur, and the fungus is then more appropriately known as *T. endo-ectothrix*. Indeed, even from the same case, hairs may be seen without any external spore sheath, or very little, and hence care is necessary in the diagnosis of this variety of trichophyton.

Further, since considerable variations occur in the size of the spores of ectothrix fungi, a hair affected with ectothrix may resemble one invaded by the microsporon. But the sheath of microsporon spores is intimately blended with the hair, and has been aptly compared by Sabouraud with the bark of a tree.

In the case of the ectothrix, a more or less distinctly sporulated mycelium is seen to lie between the hair and the wall of the follicle, extending just into, but stopping short at the mouth of the follicle, to form a sort of 'collarette.'

Mycelium between the hair and follicle is also seen in the early stages of infection by the endothrix and microsporon; as for the endothrix, such extra-pilar mycelium soon dies away, and in the case of the microsporon the threads are never in chain formation (Blaxall).

A mycelial fringe, such as is seen descending towards the bulb in the case of microsporon, has not been observed in infection by ectothrix.

In the ectothrix hair sporulated mycelial threads, or chain formations, are seen running lengthwise. In these the spores are well marked, whereas in and near the follicle wall they are often less distinct, plain mycelial threads, broken up into irregular lengths, being more obvious.

Appearance of the Cultures

Microspora cultures.—*The microspora* cultures, on *French proof agar*, are slow to develop, and by the seventh day form flat disc-like colonies beneath the surface of the medium. Small, scale-like, colonies, each with a central opaque white spot, appear on the surface by about the fourteenth day, encircled by a narrow white zone, beyond which is an irregular series of rays. The central opacity increases in size, and after about eight weeks is seen to be surrounded by concentric circles, beyond which is a slightly raised white ring; beyond this, again, extends yet another set of rays.

According to the observations of Fox and Blaxall, cultures of about six weeks' growth may be divided into four types:[1]—

(*a*) 'The first and commonest form has a slight white knob in the centre, surrounded by a greyish-white cushion, which shows here and there faint indications of concentric markings. The general aspect is, as described by Sabouraud, somewhat asbestos-like.'

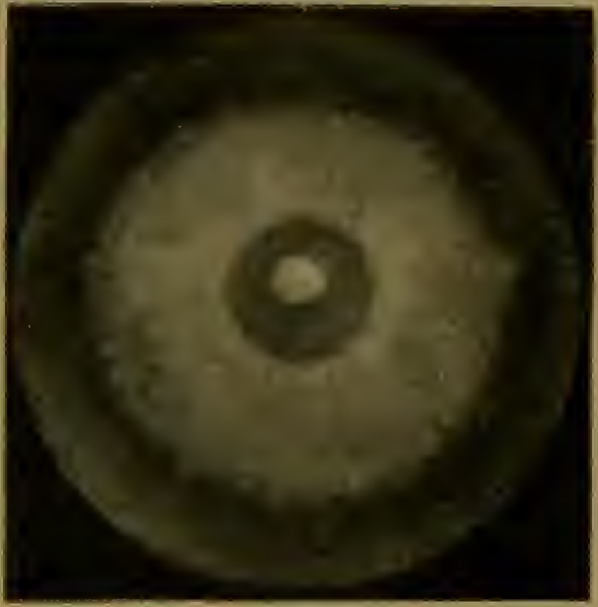

FIG. 50.—CULTURE OF A MICROSPORON AUDOUÏNI[1] ON FRENCH PROOF AGAR, THE SECOND TYPE, WITH VERY DELICATE HYPHÆ.

(*b*) 'The second kind' (shown in fig. 50) 'is a very delicate growth, and is further characterised by the microscopic appearances of the aërial hyphæ, which are extremely delicate. There is a small central boss enclosed by a clear space, and this is surrounded by a network of hyphæ which radiate to the periphery. They are more interspaced than those of the first type, and are all of a greyish-brown colour. Both the first and second types are transparent.'

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[1] See 1st footnote, p. 79. A typical collection of these cultures will be found in *Brit. Journal Dermat.* September 1896, illustrating the paper by the authors quoted.

(*c*) 'The third type shows many variations. It forms, generally within a fortnight or three weeks, a white central plaque, felt-like, with or without a central boss. The plaque may be of the size of a sixpence or a shilling, or cover nearly the whole of the plate. Its edge may be sharply circumscribed or fluted, or may extend into the periphery as a fleecy fringe. Where the plaque is circumscribed the peripheral hyphæ are relieved by a fine down, which sometimes becomes tufty. The plaque sometimes shows slight geometrical markings, or the boss may be strongly projecting.'

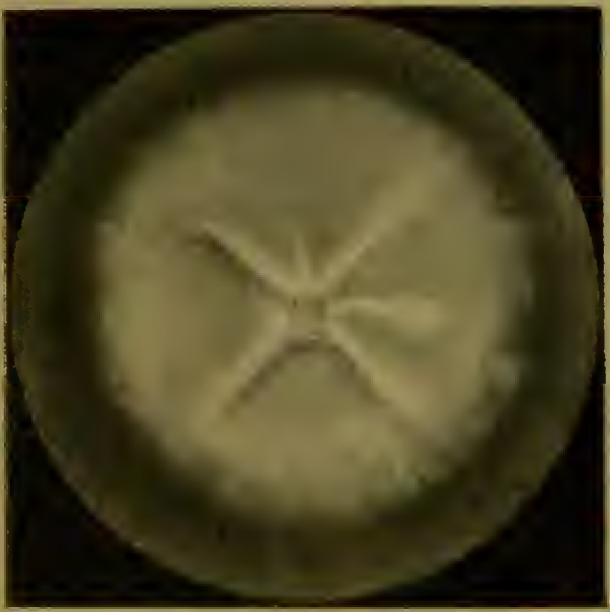

FIG. 51.—CULTURE OF A MICROSPORON AUDOUÏNI[1] ON FRENCH PROOF AGAR, THE FOURTH TYPE, SHOWING FOUR WELL-MARKED FURROWS, OR FOLDINGS, RADIATING FROM THE CENTRE.

(*d*) 'The fourth type (fig. 51) is less common. The culture is entirely covered with a luxuriant, soft, pure white down, with a small central boss. The down extends completely to the end of the submerged hyphæ, so that there is no appearance of a submerged fringe. But the main feature is that the culture shows symmetrical furrows or foldings, commonly four in number, radiating from the centre. The culture may lose its circular shape, extending along the direction of the furrows and giving a stellate appearance. These last two types, by reason of their massive growth, become opaque to transmitted light, the under surface showing a brown, felt-like appearance.

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[1] See footnote, p. 79.

'No relation is at present recognised to exist between these types, and the clinical varieties, and the different kinds of hair lesions.'

On glycerine agar, the colony forms a flat white disc, covered with down.

On wort agar, the growth appears, according to Sabouraud, as a series of superposed discs covered with down, and arranged like a cockade or rosette.

On potato, the growth of the microsporon is very typical, but slow in appearing. About the tenth day, little brown or pink spots appear, the difference in colour seeming to arise from differences in the reactions of the potatoes. At the end of a fortnight or three weeks these discolorations, likened by Sabouraud to streaks of dry blood, become the seat of aërial tufts of down, pure white in colour. The growth of this down is always much more luxuriant when the discoloration is markedly pink, pointing to a more favourable condition of the potato. With a brown discoloration the growth is feeble and appears abortive' (Blaxall).

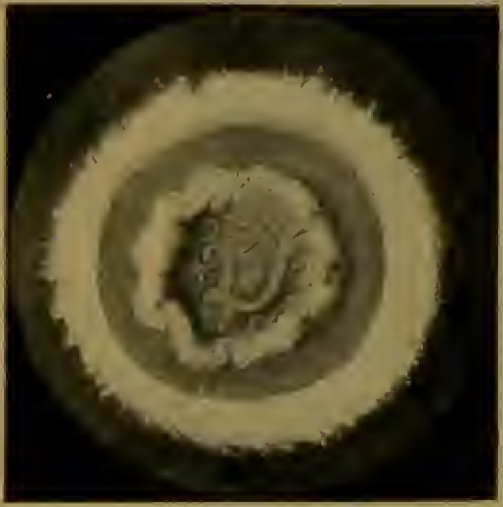

FIG. 52.—CRATERIFORM CULTURE OF A TRICHOPHYTON ENDOTHRIX,[1] ON FRENCH PROOF AGAR.

As showing the extraordinary vitality possessed by the microsporon on all the media, it is important to note that even when quite dried up, the growth has been successfully sub-cultured, by the author just quoted, from tubes two years old.

In broth, isolated mucus-like globules are seen, having a satiny lustre when seen by transmitted light.

II. Trichophyta Cultures

Sabouraud classifies the trichophyta met with in France according to their cultural peculiarities, on French proof agar, as follows :—

1. *Trichophyta with crateriform cultures*, corresponding with *T. endothrix* (three known species).

2. *Trichophyta with acuminate cultures* (having a central mound

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[1] See footnote, p. 79.

or boss), corresponding with some varieties of *T. endo-ectothrix* (three known species).

3. *Trichophyta with white powdery cultures*, corresponding with other varieties of *T. endo-ectothrix* and pure *ectothrix* (six known species).

But half of the known species of trichophyta for the present must remain ungrouped.

Adamson sums up the characters of the trichophyta cultures, as a whole, as forming submerged, sun-shaped discs, with feathery, rayed margins, and yellow, or white, *powdery* surfaces, as distinguished from the *downy* appearance seen in the microspora.

Trichophyton endothrix cultures.—There are two or three species, differing from each other only in secondary characters.

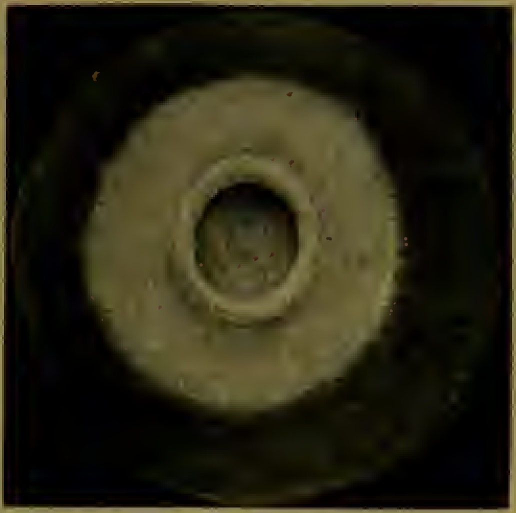

FIG. 53.—CRATERIFORM CULTURE OF A TRICHOPHYTON ENDOTHRIX,[1] ON FRENCH PROOF AGAR.

On French proof agar, the cultures are crateriform, but vary considerably in appearance, as will be evident on comparing figs. 52 and 53, drawn from plate preparations. Dr. Blaxall's description is as follows: 'Growth begins, about the sixth or seventh day, as a little star with diverging rays, more widely separated than in the microspora. The early opacity of the central part of the growth also serves to distinguish this variety from the more transparent microspora. In ten days or a fortnight the culture becomes powdery. When subcultured a heap is gradually thrown up in the centre, covered with powder, and spreading from the heap are beautifully ramified submerged hyphæ, lightly dusted over with powdery spores. In about a month's time the heap becomes depressed in the centre, as it were invaginated, leaving a central crateriform cavity. During the process of invagination, the surface of the culture cracks, leaving little groups of whitish spores against the dull brown exterior. This gives a very curious speckled appearance.'

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[1] See footnote, p. 79.

It should be noted that *on English proof agar*, the growth only slowly appears above the surface of the medium, and, though eventually covered with powdery spores, it never becomes crateriform (Blaxall).

On potato, the growth forms small powdery stars of a pure white, yellowish white, or even bright yellow colour. On these colonies becoming confluent they form a raised surface with flat top, covered with short white down. Rarely, a heaped-up growth, becoming crateriform, is seen.

On all media *T. endothrix* retains its vitality for many months, so that a subculture has been made at the end of a year. Blaxall advises the use of a considerable quantity of the growth, and it should be well rubbed into the surface of the fresh medium.

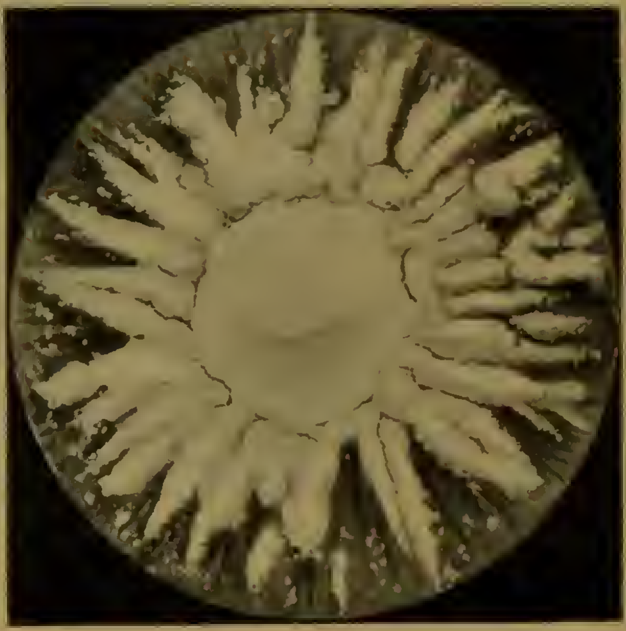

FIG. 54.—CULTURE OF A TRICHOPHYTON ECTOTHRIX,[1] ON FRENCH PROOF AGAR. Obtained from a case of pustular ringworm in a baby eighteen months old, and originating in a cat.

Trichophyton ectothrix cultures are characterised by great rapidity of growth.

On French proof agar, 'the growth commences on the fourth, sometimes even on the third day, as a white tuft, soon surrounded by a white powder, or by white hyphæ covered with a white powder. The earliest appearance is a little star with widely diverging rays, with central pasteboard opacity as in the case of *T. endothrix*' (Blaxall). Two types of cultures, at a later stage, are represented (figs. 54 and 55), and serve to illustrate Sabouraud's statement that *T. ectothrix* is always of animal origin. Though such source cannot always be traced, the exact resemblance of the cultures made from diseased human hairs to those made from conditions of ringworm found in the cat, horse, &c., under suspicion, has frequently served to prove the truth of Sabouraud's view.

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[1] See footnote, p. 79.

Fig. 54 is from a culture derived from a pustular ringworm of an infant's scalp, known to have originated in a cat. The central mound or boss, with thick radiating branches, looking as if it were 'iced' all over, is very characteristic.

Fig. 55 is from another culture, derived from a child. A slight central boss may be seen in cultures derived from the horse, but in the culture shown (also said to be exactly similar to that from a horse) the mound is absent, the centre being furrowed or corrugated.

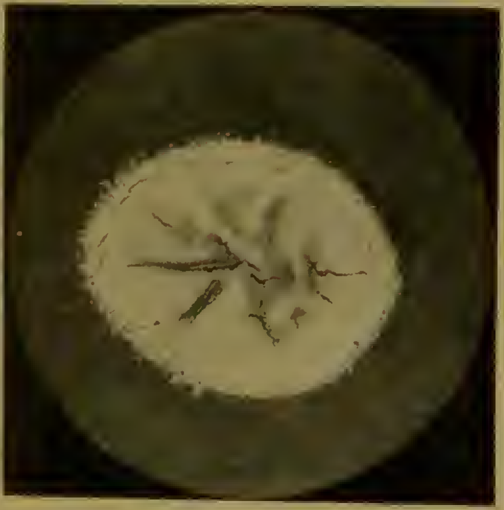

FIG. 55.—CULTURE OF A TRICHOPHYTON ECTOTHRIX,[1] ON FRENCH PROOF AGAR.
The culture is exactly similar to that from a horse.

On potato, the growth, though feebly developed, is distinctive and characterised by dry white, powdery, more or less discrete colonies, having a tendency to curl over.

Microscopical examination of cultures.—For this purpose Sabouraud advocates the use of the hanging drop, which demonstrates, he says, many points in common between a great number of the trichophyta, and also serves to distinguish these from the microspora, especially in regard to the type of fructification.

Hanging drop.—*The microsporon* culture is seen to have a mycelium composed of elongated cells, one end of each cell being swollen and club-shaped, the other end narrow. Further, curved conidiophores, bearing a row of pedunculated knobs arranged somewhat like the teeth of a comb, are described in connection with filaments of mycelium, which are really submerged hyphæ.

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[1] See footnote, p. 79.

The trichophyta present a simple mycelium without any clubbing of its component cells being visible. On examination of the hanging drop, after an interval of a few days, filaments can be seen springing from one of the original spores with which the drop was inoculated; and these submerged hyphæ then give rise in turn to a series of pedunculated spores—arranged on opposite sides along the length of the filament, which is hence called by Blaxall an 'Aaron's rod.' The spores may be nearer together, towards the end, so as to form a more or less closely set 'bunch of grapes.' This arrangement of the external spores, stated by Sabouraud to be common to all the trichophyta, led him to place them among the cryptogamic family Sporotrichum, genus Botrytis. But this appearance is, in Blaxall's opinion, more probably due to an interlacement of spore-bearing hyphæ.

Chlamydospores.—Besides the last-mentioned method of reproduction, multilocular spindle-shaped bodies, called *chlamydospores*, occur, each at the end of a filament, and they are especially developed in the white ectothrix cultures. They have never been seen in endothrix cultures by Fox and Blaxall. They are often 50 to 80 μ in length. Frequently, also, spirally twisted mycelial filaments are seen, which are probably sterile hyphæ.

Impression preparations are strongly advised by Colcott Fox and Blaxall, on the ground that the aërial hyphæ (where alone true fructification should be looked for) cannot properly develop in a hanging drop. These authors therefore consider that the appearance of fructification just described[1] in the case of the microsporon is fallacious, and that the pedunculated knobs are really only badly developed hyphæ. They have conclusively demonstrated, by means of impression specimens from plate cultures, that all known true ringworm fungi have their aërial fructification developed on the same plan, viz. a central rod bearing terminally and laterally small spores attached by a short pedicle, the so-called 'Aaron's rod,' and for this reason they regard the microspora and trichophyta as belonging to the same family.

Chlamydospores, as above described, in the case of hanging-drop specimens, are also well seen in impression preparations, and occur in the microspora and ectothrix, but never in endothrix, cultures.

Commensalism.—In the case of both microspora and trichophyta, apparently pure cultures, after being kept some time, lose their uniform appearance, little white tufts of down being seen dotted here and there. Sabouraud has distinguished these tufts by the term

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[1] See p. 91.

'*commensals*,' and has endeavoured to explain their presence on the hypothesis of their being processes of another fungus, intimately and inextricably associated with the ringworm fungus. He, with other observers, has devised various methods for the differentiation of the ringworm fungus from its commensal. Blaxall, after a close study of the subject, by means of plate cultivations made according to the ingenious method mentioned on p. 75, has come to the conclusion that this commensalism is in many cases but a pleo-morphism of the ringworm fungus itself, that the little white tufts are of the nature of a 'sport;' or, possibly, are degenerative forms, and not processes from another fungus. M. Bodin, whose careful investigations ought also to be specially mentioned, has independently arrived at the same conclusion. The whole subject is well discussed in the papers by Fox and Blaxall.[1]

Favus

Favus.—This disease is most frequently found in neglected children, and appears to be far commoner in Scotland, and Ireland, than in England. The scalp, and very rarely the nails, may be primarily affected, spreading from the scalp to the face, and then to the arms, and body generally. It may spread down the œsophagus, and in one, probably unique, case quoted by Crocker, the mucous membrane of the stomach was attacked. The entire scalp is frequently covered with circular yellow patches, called *scutula*, chiefly composed of the parasitic fungus causing the disease, and known as **Achorion Schönleini.** The scutulum grows more rapidly at the periphery than in the centre, which, remaining somewhat fixed to the deeper structures, becomes depressed and cup-like. The word 'favus' refers to the honeycomb-like appearance thus produced. The odour is very characteristic and 'mousey.' Frequently a hair may be seen escaping through each cup, and it is often decolorised to a distance of about one centimetre ($\frac{3}{8}$ in.) beyond its passage through this cup. The root of the hair is surrounded by a

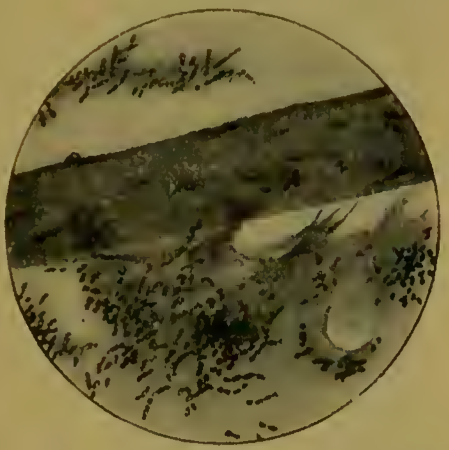

FIG. 56.—ACHORION SCHONLEINI IN HAIR AND ATTACHED SCALE. × 240
From a case of favus. Stained by Adamson's method.

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[1] *Brit. Journal Dermat.* Sept. 1896.

scale or sheath made up of the fungus in question. The presence of the *Achorion Schönleini* in the scales and hairs is readily demonstrated by Adamson's method, mentioned on p. 77.

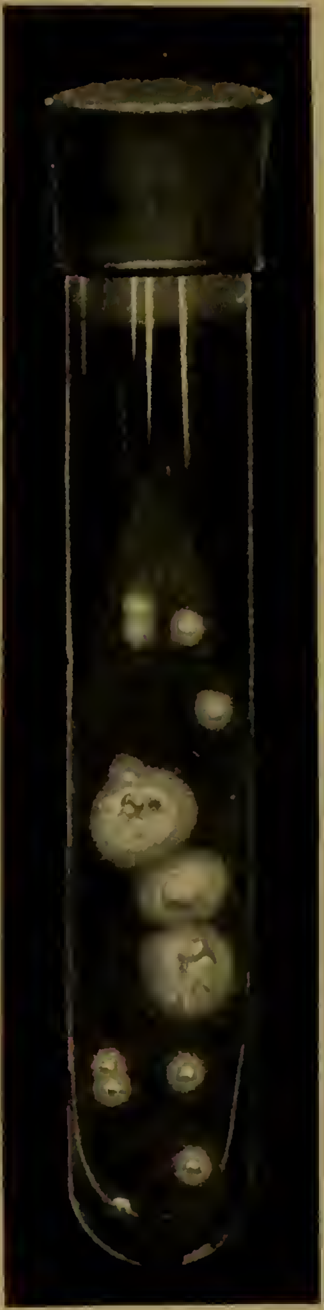

FIG. 57.—CULTURE OF FAVUS (ACHORION SCHÖNLEINI) ON GLYCERINE AGAR.

Between the younger colonies, at the upper and lower part of the medium, are seen three larger, older, wrinkled colonies.

Under the microscope, the organism is seen in the form of mycelial filaments, massed together in the hair and scales, where they are especially noticeable (fig. 56). The mycelium consists of jointed rods; many of these bear a resemblance to metacarpal or metatarsal bones, and in between two such elongated portions more spherical cells are often seen. The filaments themselves are very variable in their width, and are often sinuous. They frequently split up into three or four secondary filaments, and the point at which such division occurs has been named the '*favic tarsus*,' owing to its resemblance to the tarsus in animals (Wurtz); the sheath connecting the filaments together making up the mycelium is scarcely visible, hence the name *Achorion* (*lit.* 'without integument').

Cultivations.—The typical appearance of the young colonies is seen in fig. 57, drawn from a culture kindly placed at my disposal by Mr. Walter Severn. They are circular or oval in shape, snowy white in tint, finely powdered over the raised surface in young cultures, distinctly wrinkled in older ones. From the margin fine branches pass out in a radiating direction. In still older cultures a brownish or *café-au-lait* tint is seen, and the surface becomes very rugged, or more or less honeycombed and sponge-like. The growth is most rapid at 30°–35° C.; at 10°–25° C. the growth is extremely slow, thus differing from the trichophyta, which are readily cultivated at from 15° to 20°C. (see also p. 74). When grown on nutrient gelatine, Sabouraud says that the organism of favus further differs from the trichophyta by its power of liquefying the medium in three or four days, whereas in the case of the trichophyta liquefaction only commences on the twelfth to fifteenth day.

Although it was formerly believed that favus was always of animal origin, judging by the identical appearance of human and animal cultures, Scabrazes, and also Bodin, have demonstrated that, in most cases, the favus which develops spontaneously in the mouse is different from that of man (Wurtz). By inoculation into animals, e.g. a kitten's ear, the disease is readily produced.

Favus with trichophytoid lesions.—Sabouraud states that in France a certain number of the circinate patches on the smooth skin, exactly simulating *Tinea circinata*, and infections of the hair, simulating typical *kerion*, and in fact indistinguishable to the naked eye from those due to the *Trichophyta endo-* and *ectothrix* respectively, are really due to the organism of favus. A case of *Tinea circinata* of this kind has been recorded by Fox and Blaxall.

Under the microscope, these fungi appear in the hair either as the mycelial forms of favus, or closely resemble typical *Trichophyton ectothrix*.

The difficulty in diagnosis is further increased by the absence of typical favus cups, even when inoculated into animals.

The diagnosis is, however, readily and certainly made by means of the cultures, which are quite different from those of all known trichophyta. On the contrary, they resemble the various species of favus already known; and in addition to the characters previously enumerated it may be pointed out that, whilst the addition of saccharine bodies greatly favours the growing of the trichophyta, they do not seem to assist in the development of favus cultures, which readily assimilate nitrogenous material, organic or otherwise, in large quantities. The mode of fructification, so typical of all true trichophyta, mentioned on p. 92, is never seen in the case of the cultures derived from these ringworm-like lesions, which must therefore be included under the name of favus.

PATHOGENIC ORGANISMS

LESSON XIII

PYOGENIC ORGANISMS—SUPPURATION

The organisms of suppuration (*par excellence*)

A.—1. **Staphylococcus pyogenes aureus.**
2. „ „ **albus.**
3. „ „ **citreus.**
4. **Staphylococcus cereus albus.**[1]
5. „ „ **flavus.**[1]

B. 1. **Streptococcus pyogenes.**
2. „ **erysipelatis.**[2]

I. *Cultivations of* A (all five varieties) (i) Gelatine streak.
„ „ (ii) Agar-agar streak.
„ *of* B 1 : (i) Gelatine stab.
„ „ (ii) Gelatine streak.
„ „ (iii) Agar-agar streak.
„ „ (iv) Broth.

II. *Stain coverslips* of { A. One variety only.
{ B 1. From both fluid and solid media.
(i) With carbol fuchsin.
(ii) By Gram's method.

Gram's method, for film preparations

a. After drying and fixing the film as usual, filter on to it anilin gentian-violet, so as to completely conceal the coverslip. After five minutes, pour off the stain.

β. Without washing, pour on Gram's iodine solution.[3] Pour off after two minutes exactly.

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[1] Non-pathogenic, but introduced here for the sake of completeness.

[2] Conveniently mentioned here; though pathogenic, it is non-pyogenic; but it is probably only a variety of *S. pyogenes*. See p. 114.

[3] **The formula for Gram's iodine solution** is : iodine, 1 part ; potassium iodide, 2 parts ; distilled water, 300 parts.

γ. Without washing, place the coverslip in a watch-glass full of alcohol,[1] *until no more violet colour is seen to come away.* (N.B. This does *not* mean that the specimen is to be left in till *decolorised.*) No definite time is given, as it depends so much on the thickness of the film, and it may be necessary to transfer the coverslip to a second lot of alcohol, before the clouds of colour cease to come away. Generally speaking, the average time is from five to ten minutes.

δ. Wash in water. Dry and mount in xylol Canada balsam. Films of pure cultures cannot be counterstained, of course.

In the case of pus, sputum, &c., after washing in water and drying (stage δ, *supra*), the films should be *counterstained* in eosin as below.

III. *Stain pus* from an abscess, or from a case of 'Phlegmonous erysipelas:'—
   (i) With Löffler's blue, or carbol-fuchsin.
   (ii) By Gram's method. After washing and drying (stage δ, *supra*), counterstain with five per cent. watery eosin, for half a minute. Wash well in water, dry and mount.

IV. *Stain sections* of organs containing streptococci, *e.g.* the kidneys of a rabbit dead of acute streptococcic infection, by
   (i) Simple stains, Löffler's methylene blue, or carbol-fuchsin (see below).
   (ii) Gram-Eosin or Eosin-Gram-Weigert methods. Practise with sections cut with the ether-freezing microtome, and also with those obtained from tissues previously imbedded in paraffin.

SECTION-STAINING

The general methods for section-staining are conveniently mentioned here.

I. When not imbedded in paraffin, but cut in gum[2] with a freezing microtome.

They may be stained with the simple anilin dyes, or by Gram's method as follows:—

Simple staining

1. The section (generally preserved in 60 p. c. alcohol) is transferred to a vessel containing distilled water, and is carefully spread out on a clean glass slide insinuated beneath it.

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[1] In most of the decolorising and dehydrating processes, *e.g.* for sections, &c., unless absolute alcohol is specially indicated in the text, use strong methylated spirit, which will be found equally good, and far less expensive.

[2] For the methods of fixing, hardening, and imbedding tissues, see Appendix B, p. 273.

2. The slide and section are removed from the water, and the moisture allowed to drain away, the excess of water being mopped up with 'German blotting-paper.' The section can then be gently but firmly pressed with paper to the slide.

3. Carbol-fuchsin, or Löffler's blue, is filtered on. As a rule, the Löffler's blue is preferable. After ten minutes, in case of carbol-fuchsin, or thirty to forty minutes, in case of Löffler's blue, wash rapidly in water, drain, dry and press section to the slide with blotting-paper.

4. Dehydrate for two minutes in alcohol. Drain off alcohol.

5. Clarify in xylol, 'till a needle placed on the under side of the slide is seen through the section as clearly as if nothing intervened.'

6. Rapidly drain off, and absorb with blotting-paper, excess of xylol, and mount in Canada balsam.

The organisms are more intensely stained than the rest of the tissue, which has a similar, but fainter, tint.

Gram's method for sections.—All sections to be stained by Gram's method *must be passed through alcohol* before going into anilin-gentian-violet. The organism, unless decolorised by Gram's method, alone remains stained violet, the rest of the section being unstained. It is usual to counterstain the tissue with eosin, this being known as the *Gram-Eosin method.*

'Gram-Eosin' method of staining sections

1. The section is placed in a watch-glassful of alcohol, and transferred with a plated metal lifter to the slide. The alcohol is drained off.

2. Anilin-gentian-violet is filtered on, and after ten minutes is drained off *completely*. The section is pressed firmly on to the slide with blotting-paper,

3. Gram's iodine solution is poured on. After three minutes, exactly, without washing, it is completely drained off, and if the section seems loose, it is pressed again to the slide.

4. Pour on alcohol. Renew from time to time, *till no more colour comes away* (see p. 97). Drain off alcohol, and place the slide in water.

5. Drain off the water, and dry. If necessary, press section to the slide. Counterstain in five per cent. watery eosin for ten minutes.

6. Dehydrate in alcohol for two minutes.

7. Clarify in xylol, till the section is quite translucent.

8. Drain away, and mop up, excess of xylol, and mount in balsam. The organism, if 'taking the Gram,' is of a violet tint, the tissue being pink.

'Eosin-Gram-Weigert' method

1. Before staining in anilin-gentian-violet, counterstain the section (transferred to slide from distilled water, not spirit in this case) in five per cent. watery eosin, for half an hour. 5 min.

2. Pass through alcohol, and then filter on

3. Anilin-gentian-violet. After ten minutes, drain completely, and press firmly on to the slide; without washing, pour on

4. Gram's iodine solution. After three minutes (exactly), drain, and press firmly on to the slide.

5. Decolorise in *anilin oil*, dropped on to the slide, renewing the oil when it becomes of deep blue tint.

6. When no more blue comes away, and the section looks quite pink, remove anilin by xylol, to prevent excessive decolorisation, and, at the same time clarify.

7. Drain off xylol and mount in balsam.

Carmine-Gram-Weigert

1. Stain a section, removed from distilled water, for half an hour in lithium carmine, and place in acid alcohol (containing one per cent. HCl) for one minute, to fix the carmine.

2. Wash well in water, to get rid of the acid.

3. Pass through alcohol, and transfer to

4. Anilin-gentian-violet, Gram's iodine, anilin oil and xylol, as mentioned above.

Paraffin sections [1] (cut by the Cambridge Rocking Microtome).

1. Cleaned coverslips are held in cornet-forceps, a minute drop of a mixture of glycerine and egg-albumin (equal parts [2]) is placed with platinum loop on one coverslip, and another is superposed and then separated, so as to get an extremely thin and uniform layer on each glass.

2. Tepid water is placed in a black vulcanite tray, such as is used by photographers for quarter-plates.

3. The paraffin section is laid gently on the surface of the water, using the point of a needle, or a pair of fine forceps.

4. A narrow strip of cigarette paper is gently insinuated under the flattened-out section, which is then caught up. The paper, section-side down, is carefully laid on the prepared coverslip, which has been placed on a pad of German blotting-paper to prevent it breaking.

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[1] See Appendix B, p. 273.

[2] A drop or two of formalin, or a little thymol, should be added to prevent the growth of organisms.

5. The section is gently pressed, through the cigarette-paper, with the pulp of the middle finger, and the paper is then peeled off, leaving the section adherent to the coverslip. The water is drained off.

6. The coverslip is kept in the paraffin chamber (fig. 58), at from 50° to 60° C., for ten minutes, and then placed in a large watch-glass of chloroform, and kept covered up for fifteen minutes. If necessary, a second quantity of chloroform is used; and this will generally be found advisable.

7. The coverslip is placed in another watch-glass containing alcohol, to get rid of the chloroform. If flakes of paraffin are seen on the surface of the alcohol, the coverslip should be returned to a fresh lot of chloroform; and then, after three to five minutes, placed in alcohol.

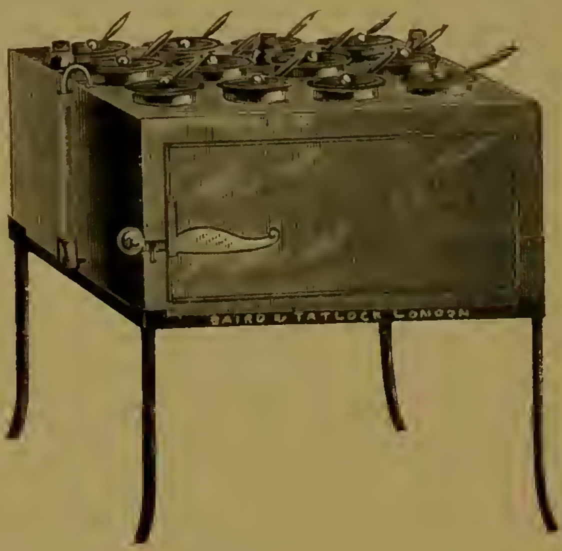

Fig. 58.—A Convenient Form of Paraffin Chamber

The little saucepans with spouts contain the paraffin, kept melted, ready for use, by the uniform temperature of the water, e.g. 53° C., in the copper jacket of the cupboard below, in which the paraffin sections may be placed, as directed in the text.

8. Wash in water, and employ methods for simple stains, or the eosin- (or carmine-) Gram-Weigert method; or, without washing in water, filter on anilin-gentian-violet and stain by 'Gram-Eosin' method. (See pp. 97-99.)

Celloidin sections[1] (cut by Schanze's, Jung's, or Minot's microtome) are generally kept in 60 p. c. alcohol till required for use. They are transferred to distilled water when simple stains are to be used, or placed in methylated spirit in the case of Gram's method. A clean slide is insinuated beneath the selected section, which is then mounted and stained, just as when using the gum-freezing method (see Appendix B), the imbedding material (celloidin), not being removed, as is found necessary in the case of paraffin sections.

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[1] See p. 274.

PATHOGENIC ORGANISMS

The practical importance of the discovery of bacteria in diseased tissues was for long discounted by many eminent authorities, who regarded this invasion of the tissues as the result, and not the cause, of the disease. Even the constant presence of the *Bacillus tuberculosis* in phthisical sputum—to give a concrete instance—was considered by many writers of vast clinical experience to be a more or less accidental association, or epi-phenomenon, determined by the more favourable conditions for the growth of the organism in question in tissues of which the normal resistance and metabolism had been already profoundly modified by disease. The opposite view, in which every disease is explained on the germ theory, is equally incorrect. The truth, as usual, appears to be midway between these extremes.

Certain diseases from their clinical history and course are obviously parasitic in origin; and in such cases, even if the search for the organism has hitherto been unsuccessful, we may safely predict its discovery sooner or later. Many of the acute specific fevers, and syphilis, are instances in point. As regards syphilis, Lustgarten has, indeed, already described a special bacillus (*Lustgarten's bacillus*), but though further confirmation of this observation is desirable, the course of the disease is such as to make it practically certain that its microbic origin will, ere long, be clearly demonstrated. So for the acute specific fevers referred to above. And the same is probably true for certain forms of malignant disease (see p. 251).

On the other hand, and for the same general reasons, it appears equally obvious that certain diseases, e.g. many of those arising in connection with the circulatory and nervous systems, are not parasitic in nature. In such cases, any associated organisms found are probably accidental in origin, or of quite secondary importance.

Before a micro-organism can be considered to be the cause of a given disease, it should conform to the four well-known **tests, or postulates of Koch**—viz.:

(i) It must always be present in the diseased tissues.

(ii) It must be capable of reproducing the disease, when injected into a susceptible animal.

(iii) It must be recoverable from the tissues of such animal, and be capable of cultivation on artificial media.

(iv) Such cultures must be capable of reproducing the disease, when inoculated into a fresh animal.

Most of the so-called pathogenic organisms have been submitted to these tests successfully, e.g. *B. tuberculosis*.

But where the disease in man is not communicable to animals, complete proof of the specific nature of the organism may not always be possible.

Sometimes there is a '**mixed infection**,' as it is called. One of the best instances of this double infection occurs in advanced cases of phthisis, with hectic fever. The *B. tuberculosis* is then associated with the *Streptococcus pyogenes*, which is responsible for the fever, and may actually be found circulating in the blood, sometimes.

When we remember the number of diseases of unknown origin, and realise that, for their rational treatment, the cause must first be found, the value of the discovery of a micro-organism causing a single disease can hardly be over-estimated. The remarkable results obtained in the treatment, both curative and prophylactic, of animals experimentally infected with certain organisms, *e.g.* of diphtheria and tetanus, should be a great encouragement to further investigation.

The organisms of suppuration

The organisms of suppuration.—Although numerous organisms have been isolated from pus, and many of these are undoubtedly pyogenic when a pure culture is inoculated, two kinds are chiefly associated with this process, and may therefore be looked upon as the organisms of suppuration, *par excellence*—viz. the *Streptococcus pyogenes*, and the *aureus* and, to a less extent, the *albus* and *citreus* varieties of the *Staphylococcus pyogenes* (fig. 59).

According to statistics, the *Staphylococcus pyogenes aureus* is much the commonest cause of suppuration, and together with the *albus* and *citreus* varieties occurs in acute abscesses generally about twice as frequently as the *Streptococcus pyogenes*; and both kinds may be present in the same abscess. Further, boils, carbuncles, and osteomyelitis are most frequently due to the *Staphylococcus aureus*, whilst puerperal fever, ulcerative endocarditis, and pyæmia are more commonly, but by no means exclusively, caused by *Streptococcus pyogenes*.

It may here be convenient to recall the meanings of the words *sapræmia*, *septicæmia*, and *pyæmia*.

Sapræmia, or *septic intoxication*, is due to the absorption of the chemical products of septic organisms multiplying locally at the seat of the injury. The poison, but not the organism manufacturing it, is in the blood, which is therefore non-infective.

Septicæmia, or *septic infection*, is due to the blood being invaded by organisms with their products. These may be so intensely toxic as to cause rapid fatal results even before any secondary local lesions have time to develop.

Pyæmia is distinguished from septicæmia by the development of secondary, or *metastatic*, abscesses, at places distant from the original source of infection, such as the lymphatic glands, joints, bones (medulla and periosteum), lungs, liver, &c.,[1] to which the organisms circulating in the blood have been carried.

To examine the blood in a case of septicæmia or pyæmia, **film preparations** may be made from a drop of blood obtained by pricking the finger. The skin of the finger, upon which a variety of the Staphylococcus pyogenes albus, S. *epidermidis albus* (Welch), commonly resides, must have just previously been carefully sterilised, by scrubbing with soap and water, turpentine, 1 in 20 carbolic acid lotion, methylated spirit—to get rid of the carbolic lotion—and finally ether is poured over it to get rid of the spirit. After allowing the ether to evaporate, the finger-tip is quickly stabbed with a sterile needle, and the films prepared, dried, and fixed by flaming as usual, or by holding over a bottle containing a solution of 1 per cent. osmic acid. Other methods are mentioned on p. 240. Simple staining, or preferably, the Gram-Eosin method, may then be employed for demonstrating the presence of cocci in the blood. Frequently, however, even in pronounced cases of septicæmia, there are so few organisms in the loopful of blood used for the film that such a stained preparation may show nothing. In such cases, cultivations of the blood should be made. A few loopfuls of blood, the above precautions for sterilising the skin having been taken, may be sufficient. As a rule a larger quantity is required.

Cultivations of the blood may be made on any of the ordinary media, but glycerine-agar is the most convenient. For the reason already mentioned, a large quantity of blood should be used, *e.g.* 3, or 5, or more c.c. Previously-poured, large 'plates' (p. 26) may be employed. Boiling tubes, containing 20 c.c. sloped glycerine-agar, will be found very convenient and more portable than plates. A large hypodermic syringe (5 or 10 c.c.), capable of being sterilised by boiling, is necessary. It is taken direct from the steriliser, and as soon as needle and barrel are cool, the needle is plunged through the sterilised skin[1] into a large vein, such as the median cephalic, rendered prominent, as for blood-letting purposes, by a proximally placed bandage. Five, or more, c.c. can then be withdrawn, and at once injected into the large agar tube, or prepared plate.

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[1] Prior to the formation of actual abscesses, there must be areas temporarily, at least, in a condition of non-suppurative inflammation, due to secondary deposits of the circulating organism and the local manufacture of its toxin. Lesions produced by general infection with the *gonococcus* (p. 123), for instance, may never proceed to suppuration. But in spite of this, gonorrhœal arthritis, endocarditis, &c., ought, perhaps, to be considered as pyæmic, rather than as septicæmic, manifestations.

Besides the varieties of the staphylococcus and streptococcus, to be again referred to, the following are amongst the most important of the organisms which have been isolated from pus; and most, if not all, of these appear to be definitely pyogenic in nature. With the exception of *B. aërogenes capsulatus*, they are dealt with at greater length elsewhere in this work.

Pneumococcus of Talamon-Fränkel, with which the *Micrococcus pyogenes tenuis*, also found in pus, is thought to be identical.

Diplococcus intracellularis meningitidis of Weichselbaum,[2] said to be present in the interior of the pus cells in 50 per cent. of cases of **epidemic cerebro-spinal meningitis.** It is decolorised by Gram's method.

Gonococcus of Neisser.

Micrococcus tetragonus.

Pneumobacillus of Friedländer.

Bacillus aërogenes capsulatus, a non-sporebearing, and encapsuled, rod-shaped organism, as thick as an Anthrax bacillus, occurring in short or long chains. It is responsible for certain cases of **emphysematous gangrene**, where there has been bruising of the tissues, followed by the development of much gas. It is stained by Gram's method.

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[1] If done suddenly but steadily, taking care to keep the point of the needle of the 10 c.c. syringe always within the lumen of the vein, the pain is barely perceptible, to judge from personal experience.

Intravenous injection in the case of animals may be carried out on the same general plan, the site selected for inoculation being generally one of the prominent veins near the lateral margin of the ear, a fine hypodermic needle, however, being necessary (see footnote, p. 152).

[2] A very similar diplococcus, and likewise decolorised by Gram's method, has been shown by Dr. G. F. Still (*Journ. Path.*, and *Path. Soc. Trans.*, 1898) to be the cause of simple, or **non-tuberculous, posterior basic meningitis**, a disease chiefly occurring within the first year of life, first described by Drs. Gee and Barlow in 1878; and afterwards, more fully, in 1897, by Dr. Carr, and by Dr. Barlow and Dr. Lees. 'This diplococcus, easily distinguished from the pneumococcus by culture and by inoculation, is very small, averaging in the meningeal exudation 1·2 μ to 1·5 μ. The two cocci of which it consists have their opposed surfaces more or less flattened, and are separated by a narrow, clear, space. There is a marked tendency, especially in cultures, to grouping in pairs, side by side, producing an appearance like that of a tetracoccus. Its vitality is greater than that of the pneumococcus, or of the diplococcus intracellularis, but it is less virulent than either.' (Still, in Allchin's *Manual of Medicine*, vol. i., 1900).

B. prodigiosus.

B. pyocyaneus. (See p. 42.)

B. tuberculosis, which is, however, very frequently indeed associated with the *Streptococcus pyogenes.*

B. typhosus, and *B. coli*, the latter being the cause of the fatal peritonitis which may supervene in a case of strangulated hernia.

B. anthracis.

B. mallei (Glanders bacillus).

Actinomyces—the ray fungus—is often associated with suppuration, and in cases of actinomycosis it may be obtained in pure culture, or mixed with the *Staphylococcus pyogenes aureus.*

The Amœba coli (p. 249) is also frequently found in the pus from cases of tropical abscess of the liver, associated with dysentery. In this connection, however, it should be mentioned that it is now well established that pus from liver abscesses may be sterile on cultivation. The explanation usually given is that the pyogenic organisms, having done their work, have been killed (and subsequently disintegrated) by contact with bile, which has germicidal properties.[1]

'Aseptic suppuration.'—This brings us to the vexed question as to whether suppuration can ever be produced, *e.g.* by chemical or other agencies, apart from the presence of organisms. It is obvious that the action of harmful bacteria, terminating in suppuration, must ultimately result from the chemical products of their metabolism, or from the chemical materials of which they are themselves composed. So that, on *a priori* grounds, *aseptic suppuration* is theoretically possible, and has, in fact, been experimentally produced. Needless to say, the utmost precautions against sepsis have to be observed in such experiments, and for a positive result it is necessary to select both the animal and the chemical irritant. Thus mercury, or a 5 per cent. solution of silver nitrate, produces suppuration in the dog, but not in the rabbit, whilst ammonia has no effect in either (Hewlett). Sterile turpentine (♏ xx), injected subcutaneously in the case of a young subject,[2] produced a local necrosis of the tissues, the 'abscess' containing neither pus cells, microscopically, nor organisms of any kind, as tested by culture.

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[1] See, however, Cantlie's views, p. 251.

[2] This negative result is otherwise of some interest, as it was obtained in a case of typhoid fever (before the introduction of Widal's sero-diagnosis test). The injection was made by the author in the hope of confirming the statement that typhoid bacilli are to be found in such 'abscesses.'

The view generally held at present, therefore, is that, though aseptic suppuration may be produced in the laboratory by injecting chemical substances under certain circumstances, it must be looked upon as a pathological curiosity rather than as a normal occurrence. In the vast majority of cases, clinically, suppuration results only from the presence of micro-organisms.

Staphylococcus

There are at least five well-known species of staphylococci, viz:

1. **S. pyogenes aureus.**
2. ,, ,, **albus**—of which the *S. epidermidis albus*, of Welch, appears to be a variety (p. 108).
3. ,, ,, **citreus.**
4. **S. cereus albus** } non-pyogenic.
5. ,, ,, **flavus** }

1. **Staphylococcus pyogenes aureus** is widely distributed, being found in the air, water, and soil. It occurs normally on the skin and in the respiratory passages. It is the commonest cause of suppuration, and is met with in acute abscesses (see p. 102), boils, carbuncles, and osteomyelitis; also—but less commonly than the *Streptococcus pyogenes*,—in septic endocarditis, puerperal fever, and pyæmia. In the last-mentioned condition the organism spreads through the system by the circulation, and metastatic abscesses are produced in various bones and joints, the lungs, liver, and other organs. It occurs very commonly in tonsillitis, especially in 'Septic throats,' alone, or mixed with streptococci, &c. Cultures from diphtheritic throats very frequently contain staphylococci, and streptococci, as well as, of course, the characteristic Klebs-Löffler bacillus.

Cultivations.—*Gelatine stab.*—A white streak appears along the track of the needle in less than twenty-four hours. Liquefaction of the medium occurs rapidly; in most cases, by the end of a few days. The growth, which soon assumes an orange tint, sinks to the bottom of the tube. A variety of this organism does not liquefy gelatine; and, in secondary cultivation, the orange colour may be replaced by a light buff, or dirty white, tint; so that distinction from S. pyogenes albus may be, at first, difficult.

Agar-agar streak.—The colonies are discrete and circular, and of an orange tint, especially marked in the raised central point. The colonies may, however, fuse together to form an orange-coloured streak. The orange colour, often more pronounced when the agar has been kept at 20° C., may disappear, more often perhaps than is the case with gelatine cultures; in fact, transference from agar to gelatine may restore the orange tint to some degree.

Serum.—The growths are similar in appearance to those seen on agar-agar.

Milk is rapidly coagulated (in a few days).

Under the microscope, with a low power, a finely dotted network can be made out occupying the centre field. With the oil-immersion lens, this is seen to be made up of chains of cocci, some single, others in pairs, or in longer chains. The most characteristic groups, however, are composed of triangular, pear-shaped, or diamond-shaped masses of cocci (fig. 59 B), often arranged in a series of planes, lying one on top of the other, so as to present, on focussing up and down, a very solid appearance, compared with a bunch of grapes (σταφυλή, a bunch of grapes).

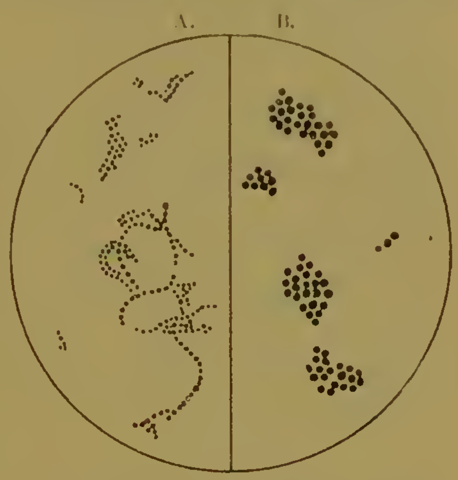

Fig. 59

A. STREPTOCOCCUS PYOGENES. B. STAPHYLOCOCCUS PYOGENES AUREUS.

× 1,000

(Drawn from a microphotograph lent by Prof. Sidney Martin, F.R.S.)

The size of the cocci varies considerably in different cultures of the same varieties of organism; and active multiplication, indicated by the presence of elongating ovoid cocci, obviously undergoing division, may frequently be seen.

The pathogenic properties[1] of this organism, though it is met with so universally, may be very simply demonstrated by rubbing a pure and virulent culture, e.g. from an acute abscess, into the skin of the arm, after this has been thoroughly sterilised. A crop of boils, from which the organisms can be recovered, will result. Another familiar instance is also seen in the boils so frequently occurring at the back of the neck, when the skin has been chafed by a stiff collar.

The effect of inoculating susceptible animals with the staphylococcus pyogenes aureus.—Rodents, and especially rabbits, are the most suitable for this purpose.

Subcutaneous, or even *intrapleural*, or *intraperitoneal*, *injection* is frequently followed by a negative result, unless the tissues indicated have been previously injured, or the circulation damaged. So also in the case of *intravenous* injection, a small or sub-fatal dose circulating in the blood without giving rise to an abscess.

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[1] Professor Sidney Martin, in 1892, showed these to be due to a fever-producing albumose, and a basic body, present in the blood and spleen.

Where, however, the culture is very virulent or the tissues damaged, a rapidly fatal, suppurative inflammation, or general septicæmia, may follow in from twelve to forty-eight, or seventy-two, hours. Such a condition of staphylococcic infection, or *staphylomycosis*, spreading through the lymphatics so as to reach the general circulation, may result in a condition of staphylococcic pyæmia, or '**metastatic staphylo-mycosis**' (Bulloch).

A localised abscess results, in the case of *damaged soft tissues*; or suppurative periostitis, or osteomyelitis, in the case of *bone*. The organisms in the blood-stream are arrested in the injured tissue, in which they can multiply and develop their poisonous products more favourably than when they are fully exposed to the microbicidal effects of the phagocytes, plasma, &c., in the circulating blood. Damaged tissue may be looked upon as an area to a greater or less extent deprived of its normal protective mechanism.[1]

In the case of a joint, complete destruction of the articulation, or even death, may rapidly supervene upon the injection into it of virulent culture.

Staphylococcus pyogenes albus.—Except for the colour remaining permanently white, instead of changing to an orange tint, most of what has been said of S. aureus may be said of the albus variety. It is perhaps less frequent than S. aureus, but in cultures taken from throats of suspected cases of diphtheria, each series of cases extending over a period of two or three months, the present writer has sometimes seen the albus variety in the large majority, S. aureus being practically absent altogether. According to Watson Cheyne, S. albus is more virulent than S. aureus, the reverse opinion having been generally held hitherto. Double infection with the two varieties is thought to be more serious than when the aureus alone is present.

Cultures present very much the same appearance as those already described, liquefaction of the gelatine generally occurring, but it may be delayed, or entirely absent. The colonies are opaque white, or porcelainous, with irregularly rounded edges, the central portion of the colony being more opaque than the periphery. A continuous streak may be produced by the confluence of the discrete colonies.

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[1] This is a point of practical interest; and, indeed, it is for this reason that the authorities at the Pasteur Institute at Paris so strongly condemn the practice, so common in England, of applying lunar caustic—rather than the antiseptics in ordinary use—to wounds resulting from the bites of rabid, or supposed rabid, animals. Such cauterised tissue forms a slough in which any virus, yet remaining in the deeper part of the wound, may thrive to a dangerous extent, because the slough with its contained virus is *practically* non-vascular. The natural defensive agencies in the blood, therefore, cannot reach the seat of infection, until, perhaps, too late to be of any use.

Staphylococcus epidermidis albus (Welch) is thought to be only a less virulent variety of S. pyogenes albus ; it is said to be the commonest organism found on, and in, the skin, and is frequently found in healing wounds. It has distinctly less pyogenic properties, it liquefies gelatine more slowly, and the colonies on agar are generally smaller than in the case of the ordinary variety, S. pyogenes albus.

It is the cause of the stitch abscesses so commonly seen when there has been excessive tension.

Remembering how commonly it and the other varieties of staphylococci occur on the skin normally, the need for careful sterilisation of the integument when examining the blood in cases of septicæmia, &c., must be obvious.

Staphylococcus pyogenes citreus.—This is not infrequently seen in septic conditions of the throat, and (associated, of course, with Klebs-Löffler bacilli) in diphtheria.

Cultivations on *gelatine*, *agar-agar*, or *serum* : in each case a polished, lemon-yellow growth appears. Gelatine is liquefied. The organism is pathogenic to guinea-pigs and rabbits. Apart from the colour, its cultures on the different media are similar to those of the S. aureus and albus.

Under the microscope, it is indistinguishable from the other varieties of pyogenic staphylococci.

Staphylococcus cereus albus. Staphylococcus cereus flavus.—These two organisms, though found in abscesses, are apparently non-pathogenic, to judge by the negative result of inoculation experiments.

Cultivations.—The general aspect of the cultures which are, respectively, of white and yellow tint, resembles those already described, but the surface of the colonies on gelatine, instead of being somewhat moist and glistening, are dry and waxy in appearance, and have therefore been well compared with drops of stearine, or of sealing-wax. Further, the gelatine is not liquefied in either case.

S. cereus flavus differs from the corresponding *S. pyogenes citreus* in becoming of a somewhat darker tint—citron—not lemon-yellow colour. But this distinction is not always to be readily made out in sub-cultures.

Under the microscope, the cereus and pyogenic varieties of staphylococci are identical in appearance.

Streptococcus

This organism has been generally divided into two chief species, called *Streptococcus pyogenes*, and *Streptococcus erysipelatis*, the one being concerned in producing suppuration, the other being found in cutaneous erysipelas. There is, however, a considerable amount of evidence [1] in favour of the more modern view, that these two are not distinct species, but varieties of one organism which have acquired different properties according to their virulence, the position in which, and the conditions under which they have been grown for several generations (see p. 114).

Postponing the discussion of this question for the moment, we will consider the ordinary Streptococcus pyogenes.

Streptococcus pyogenes.—This organism is found in the acute forms of suppuration (cf. p. 103), in many cases of acute septicæmia, pyæmia, and puerperal fever, in septic throats associated with diphtheria, in suppurative pleurisy and peritonitis, bronchitis, in the bronchopneumonia following measles, scarlet fever, &c., and as a secondary infection in phthisis. The febrile condition known as hectic in the last-mentioned disease is due to the presence of the streptococcus, which, in advanced cases, may be found actually circulating in the blood (Jakowski). It may be found in the mouth, and respiratory passages generally, in perfectly healthy individuals; and although its virulence in such a case has been so completely reduced that it is perfectly harmless, by repeated inoculations into a series of susceptible animals, preferably the rabbit, its virulence may be restored, so that such an animal may be killed in less than twenty-four hours with a very small dose. The nature of the chemical products formed by the Streptococcus pyogenes in liquid media has not yet been accurately made out (see p. 115). In spite of our ignorance in this respect, horses and other animals have been immunised against infection with the organism by a succession of sub-fatal doses, until at length they will stand as

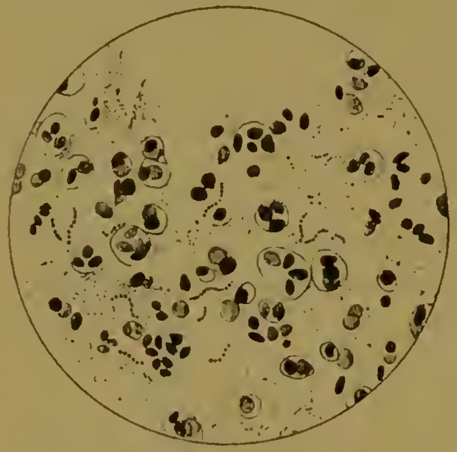

FIG. 60.—STREPTOCOCCI IN PUS, FROM A CASE OF PHLEGMONOUS ERYSIPELAS. × 530.

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[1] See Dr. Bulloch's valuable paper 'The rôle of the Streptococcus pyogenes in Human Pathology,' *Lancet*, April 11, 1896.

much as a thousand ordinarily fatal doses, or, in other words, a dose a thousandth part of which would kill a horse not treated in this way. Moreover, the animal is not only protected from the effects of a fatal dose, but its blood-serum in the same way is a powerful prophylactic for other animals and man.

After the actual onset of acute septic mischief associated with the presence of the Streptococcus pyogenes, e.g. in puerperal fever (when streptococcic in origin), such **anti-streptococcus serum** may be used as a curative agent. Bulloch has shown that an animal immunised against the Streptococcus erysipelatis is also immune against the Streptococcus pyogenes—a great argument in favour of the view that these two organisms are really identical.

It is a well-established clinical fact that a sharp attack of erysipelas has a marked effect in checking, sometimes even curing, associated sarcomatous disease.

Coley's fluid is made by growing *B. prodigiosus*, for about ten days, in a ten days' old broth culture of very virulent *Streptococcus erysipelatis*. The fluid is sterilised by heating to 60° C. for an hour, and putrefaction prevented by the addition of thymol.

It has been used with apparently permanent success in a few cases of sarcoma and carcinoma, especially spindle-cell sarcoma. Many failures have, however, been recorded, and it must also be remembered that tumours of 'undoubtedly sarcomatous' nature, clinically, not infrequently entirely disappear spontaneously. This treatment is, therefore, best suited to otherwise inoperable cases, as its author insists.

Cultivations.—(Streptococcus pyogenes and erysipelatis) —*Gelatine stab.*—The growth, which attains its maximum in three or four days, appears along the track of the needle in the form of opaque white spherical dots, about the size of an ordinary pin's head. There is no liquefaction of the medium.

Gelatine streak.—The growth consists of minute white dots, sometimes compared with minute dewdrops. The tube should be held up to the light in order to see the growth well. It does not tend to spread laterally.

The variations in the appearance of S. pyogenes and S. erysipelatis on this medium are too slight and inconstant to be of any diagnostic value.

Agar-agar streak.—The colonies, which generally appear at the end of twenty-four hours, present much the same white, or greyish, appearance as on gelatine, but the colonies are more translucent and less easily seen.

Blood serum.—The colonies are scattered freely over the surface, and are somewhat more raised than on the other media. The dotted appearance due to minute white colonies is very characteristic.

Broth.—In twelve hours turbidity of the medium occurs, but this passes off in a day or two, the growth sinking to the bottom as a white deposit, leaving the supernatant fluid quite clear, and of acid reaction.

Milk is coagulated in four or five days' time. In some cases, however, no coagulation occurs.

Under the microscope, the organism is seen (figs. 59 A. and 60) to consist of cocci, singly, and in twos, and fours, and long chains, the cocci being arranged in Indian file, the longer threads being curved irregularly. Such elongated, curvilinear chains are frequently better seen in liquid than in solid media cultures. They are also well seen in pus from pyæmic abscesses.

Multiplication generally occurs in the following way: the coccus elongates in the direction of the length of the chain. It then splits across at right angles to that direction, and so two cocci are formed. This accounts for the variation in the size of individual cocci. The elongation may, however, take place at right angles to the direction of the thread, and the splitting will then occur in the direction of the length. In this way a chain of streptococci may be seen in part duplicated (see fig. 59 A). In addition, a chain of cocci, arranged in Indian file, may here and there become spread out into a triangular mass, thus simulating a growth of staphylococcus; and when the preparation is made from a culture on a solid medium, the distinction may occasionally be somewhat difficult. An examination of the whole field in the case of streptococci will show, however, numerous short or long chains of cocci arranged in single line. In the case of the streptococcus, also, the transference of the growth to broth will generally result in the production of long thread-like forms which prevent any confusion with the staphylococcus.

Occasionally, the *pneumo-coccus* occurs in more or less elongated chains, sometimes curved so as to form almost a complete circle. In pneumococci chains, the cocci are arranged in pairs, the interval between succeeding pairs being greater than between the individuals of each pair. In the case of streptococci, the individuals follow one another closely, no distinction into pairs being evident, as a rule.

In film preparations of pus (fig. 60), from an acute abscess, stained simply, or by the Gram-Eosin method, the streptococcus may be seen in pairs or short chains; occasionally very long threads are present. Most of the organisms are seen to lie between, or on the surface of, the pus cells; but a good many cocci are contained within the cells, by which they have been absorbed in a living or dead condition (*phagocytosis*).

In sections of the viscera from an animal, *e.g.*, a rabbit inoculated with the Streptococcus pyogenes, large numbers of cocci will be found in the blood vessels, in places massed together to form thrombi. In the tissues around, paler, badly staining areas of necrosis may be seen.

The effect of inoculating susceptible animals, e.g. rabbits, with the Streptococcus pyogenes varies considerably. Thus, 'a transient redness at the point of inoculation may be all that is seen. At other times there may be a severe erysipelas, or a phlegmon. The animal may die with a large local lesion, or death may ensue from a rapidly fatal septicæmia, while locally nothing may be found, or at most a slight gelatinous-like œdema. It is also known that streptococci from severe fatal human cases may have only a slight effect on the ordinary, experimentally susceptible, animal (mouse, rabbit); whilst cocci from mild cases in man may, from the outset, be extremely pathogenic for these animals.'[1]

Generally speaking, the effect of inoculation depends upon the source, *e.g.* whether derived from a case of erysipelas, or from an acute abscess, and upon the virulence[2] and the seat of injection. The addition of even a filtered culture of typhoid bacilli greatly exalts the virulence of streptococci.

Injected into the marginal vein of the ear (the vein usually selected), a virulent culture of Streptococcus pyogenes may cause death in twenty-four hours, or even less. The organisms are then found in the heart blood, and in most of the viscera, liver, spleen, and kidneys; and in preparations of these organs, they are seen principally in the vessels.

Injected subcutaneously, S. pyogenes, and still more the S. erysipelatis, may set up a localised, sharply defined, erysipelatous rash, with no tendency to pus formation. **In cutaneous erysipelas**, as also in this experimental result, the streptococci will be found in the lymph spaces, and in the dilated lymphatics within, and just beyond, the sharply defined, red margin, *i.e.* in the lymphatics of apparently healthy-looking skin, the spread of the rash being preceded by that of the organisms.

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[1] Bulloch, *Trans. Brit. Inst. Prevent. Med.*, First Series, 1897.

[2] The enormous *exaltation in virulence obtained by passage through a series of rabbits* is well shown by Bulloch (*loc. cit.*), who obtained from a bad case of facial erysipelas an ascitic fluid-broth culture of streptococci, of which the minimal fatal dose was originally one-tenth c.cm. After twenty-six passages through animals, a fatal result was obtained by the use of *only one millionth of a cubic centimetre.* Other workers have confirmed this result.

Injected into the cellular tissues, S. pyogenes, as also S. erysipelatis under favourable conditions, such as passage through several animals in this situation, may set up a phlegmon, or phlegmonous erysipelas, a localised but distinctly pyogenic condition.

Either condition of streptococcic infection, or strepto-mycosis, may become generalised, so as to result in a streptococcic pyæmia, or, as Bulloch prefers to call it, a **metastatic strepto-mycosis**. In such a general infection the streptococcus travels along the lymphatics, thereby setting up lymphangitis, and inflammatory affections of any serous membranes in its path, until at length the circulatory system is reached.

Varieties of Streptococci hitherto described.—Six species, divided into three groups, have been described.[1]

Group I., pathogenic to rabbits, guinea-pigs, and mice, includes the *Streptococcus pyogenes*, *S. pyogenes malignus* (isolated by Flügge from necrotic foci in a leucocythæmic spleen), and *S. septicus*.

Group II., non-fatal, and as a rule non-pyogenic, but setting up erysipelatous affections when subcutaneously injected into rabbits and mice – includes the *Streptococcus erysipelatis and S. septopyæmicus*. These are, however, believed to be identical.

Microscopically, Groups I. and II. are not to be distinguished.

Group III. contains only one species, rapidly fatal to rabbits and mice (but not to guinea-pigs). From its setting up a general suppurative arthritis, after intra-venous inoculation, in the case of rabbits, the joints being distended with pus teeming with this organism, it is known as the *Streptococcus articulorum*.

Under the microscope, it is distinguishable from Groups I. and II., as it forms long threads, made up of numerous segments, each consisting of two little hemispheres, with a delicate, clear, intermediate zone. It was isolated by Löffler from diphtheritic throats.

Relation between the foregoing varieties of Streptococci.—As already indicated, it appears to be highly probable that these varieties are really not distinct species. Experimental evidence has led recent investigators, notably Dr. Bulloch,[2] to regard them as identical. The author last quoted concludes that there are 'four main factors the variations of which determine the nature and extent of streptococcic lesions:

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[1] Sims Woodhead, in Treves's *System of Surgery*, vol. i. pp. 27–29.

[2] The rôle of the Streptococcus pyogenes in Human Pathology, *Lancet*, 1896. See also papers by the same writer in the *Reports British* (now, *Jenner*) *Institute of Preventive Medicine*, 1897–1898.

'1. The first and most important—*the extraordinary variability in the virulence* of the microbe. From the streptococcus harmlessly inhabiting the mouth, we may pass experimentally through all stages of virulence, till we obtain an organism of such exalted virulence as to be capable of producing a rapid and general infection. (**Bacteriæmia.**)[1]

'2. *A variation in the site and depth of the infection* produces a difference in the nature and severity of the lesion. This seems to explain why at one time an erysipelas is developed, and at another time a phlegmon.

'3. *Varying resistance on the part of the body*, e.g. the severe streptococcic lesions met with in advanced cases of diabetes and Bright's disease.

'4. *The association of Streptococcus pyogenes with other microbes*, pathogenic and non-pathogenic. Such mixed infections are conveniently termed, by Verneuil, **polymicrobic infections**.

Most interesting observations have been made in confirmation of the second statement, all tending to prove the identity of the *Streptococcus erysipelatis* with *S. pyogenes*. *S. erysipelatis*, if inoculated subcutaneously, sets up erysipelas, and is usually non-pyogenic. But after repeated injections at greater depths, it at length acquires pyogenic properties, giving rise to suppuration, and not to erysipelas. The converse experiment has also been successful.

Further, Bulloch has shown that an animal immunised against the one species of streptococcus is immune also against the other.

The exact nature of the products secreted by the streptococcus, though presumably a soluble and very powerful poison, is not yet known. Filtrates of anaërobic broth cultures are said to have proved fatal to rabbits. These animals have been rendered immune by injecting filtered cultures previously heated to 120° C. (Roger). Gradually increased doses of *living cultures* should, however, be used in preference to the filtered toxine.

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[1] See second footnote, p. 113.

LESSON XIV

THE ORGANISMS OF PNEUMONIA, GONORRHŒA, &C.

A. **Pneumococcus of Talamon-Fränkel**
B. **Pneumo-bacillus of Friedländer**
C. **Gonococcus of Neisser**
D. **Micrococcus tetragonus**

I. *Cultivations*

of A. The Pneumococcus, on (i) Gelatine streak (no growth below 24° C.).
(ii) *Blood-agar streak* (i.e. Glycerine agar streaked, under aseptic precautions, with blood, and kept at 37° C. for twenty-four hours, to see if it is sterile before use).
(iii) Blood serum. (iv) Broth.

of B. The Pneumo-bacillus on (i) Gelatine stab.
(ii) Gelatine streak. (iii) Agar-agar streak.

of C. The Gonococcus in pus on (i) Blood-agar (made as above).
(ii) Blood serum.

of D. Micrococcus tetragonus, on (i) Gelatine stab.
(ii) Gelatine streak. (iii) Agar-agar.
(iv) Potato.

II. *Stain coverslips*

of A. *α. Culture*: (i) with carbol-fuchsin.
(ii) by Gram's method.
β. Pneumonic sputum. Use the Gram-Eosin method.

of B. *Culture*: (i) with carbol-fuchsin.
(ii) by Gram's method (Film decolorised).

of C. *Culture, or pus*: (i) with Löffler's methylene-blue, for five to ten minutes, and wash well.
(ii) with Löffler's methylene-blue, for one minute, and wash till most of the blue disappears. Counterstain, for twenty to thirty seconds, in watery eosin. Wash, dry and mount.

of D. *Culture*: (i) with carbol-fuchsin.
(ii) by Gram's method.

The Pneumococcus

Pneumococcus of Talamon-Fränkel.—The cause of acute croupous pneumonia is now known to be a diplococcus, which has been given many names, as the following list will indicate:

Diplococcus pneumoniæ of Weichselbaum.

Micrococcus lanceolatus of Talamon.

Micrococcus of sputum septicæmia of Fränkel.

Microbe de salive of Pasteur.

Micrococcus Pasteuri of Sternberg, or *Sternberg's micrococcus.*

Friedländer's pneumo-bacillus was at one time credited with being the cause of pneumonia, but this organism appears to be the real cause in only a small minority of cases. In Vienna, at one time, 7 per cent. of the cases were thought to be primarily due to Friedländer's pneumo-bacillus, whilst 85 per cent. were attributed to the pneumococcus of Talamon-Fränkel, the remaining 8 per cent. of cases being due to various other organisms, *e.g.* strepto-, and staphylo-cocci, B. typhosus, B. anthracis, &c.

Most modern writers, however, appear to agree that the great majority of cases of acute croupous pneumonia are primarily due to the pneumococcus of Talamon-Fränkel, the other organisms above mentioned playing only a secondary part. The pneumococcus is also responsible for some cases of lobular pneumonia, but the majority are caused by other bacteria, notably the streptococci (Washbourn).

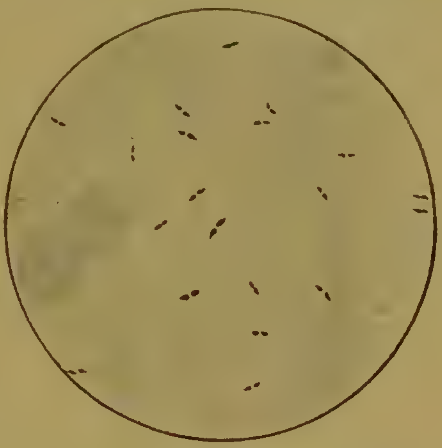
FIG. 61.—DIPLOCOCCUS PNEUMONIÆ IN PLEURITIC EXUDATION. × 925
From mouse inoculated with rusty sputum.

The pneumococcus was first isolated from the interior of the healthy mouth.

If the sputum from a case of acute lobar or croupous pneumonia be examined, it will be found teeming with pneumococci; and if a loopful is mixed up with a little broth and injected subcutaneously into a guinea-pig or mouse, death may occur in less than twenty-four hours, with a serous exudation into the peritoneal and pleural cavities, from which exudation, as also from the heart blood, pure cultures can readily be obtained.

Cultivations.—*Gelatine stab.*—There is no growth in this medium below 24° C., forming a very marked contrast to the exuberant and characteristic culture obtained in the case of Friedländer's pneumobacillus.

Gelatine streak or plates.—Owing to the low temperature requisite for this medium, the growth does not ordinarily occur at all. The pneumococcus can, however, be grown at 25° C. if the gelatine in the medium is raised to 120 per cent. Under these conditions, the colonies are sharply defined, very slightly raised, white, and finely granular points. There is no liquefaction of the gelatine.

Glycerin agar-agar.—The colonies appear as discrete, white points dusted over the surface of the medium. At first they may be seen only with considerable difficulty, best by inclining the surface of the medium, so as to obtain oblique illumination. They are more minute, and less raised from the surface, than those of the *Streptococcus pyogenes* or *erysipelatis*.

Blood agar-agar.—This medium, which is one of the most favourable for the growth of pneumococcus, is made from the preceding by the addition of a few loopfuls of blood taken from a finger-prick, after previous sterilisation of the skin, or from an animal immediately after death. The tubes, prevented from drying by rubber caps, are placed in the incubator at 37° C. for three days; if they remain sterile, they then are ready for use. The colonies on this medium are similar to those already described, except that the whiteness of the growth is obscured by the surrounding blood. Should there be any moisture collected in the bottom of the tube, capsulated organisms may frequently be obtained, even in culture.

Blood serum.—This is also a very favourable medium. The growth consists of minute, slightly raised, white colonies dotted over the surface of the medium. There is little or no tendency for individual colonies to increase in size, as in the case of some other organisms—e.g. *B. diphtheriæ*, which might possibly be mistaken for the pneumococcus at the very early stage. Capsulated organisms may be obtained from the clear serum collected at the bottom of the tube in many cases.

Broth.—Turbidity due to growth appears after twelve hours' incubation at 37° C. Subsequently the medium becomes clear as the growth becomes deposited at the bottom of the tube. Washbourn, who has recently made an extensive study of this organism, recommends[1] that nutrient broth should be sterilised at a temperature not exceeding 60° C. He insists upon the great importance of having a strictly alkaline medium.

Potato.—No growth occurs. *Milk* is coagulated.

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[1] *Trans. Path. Soc.*, London, 1895.

Vitality and virulence.—The organism shows the greatest variability in regard to its vitality, agar cultures generally dying within a week, and those of broth within two or three days. Washbourn mentions exceptional instances where broth cultivations, twenty-three days old, injected into the peritoneal cavity, produced death in four days, the blood being crowded with pneumococci; and, in the case of agar, the growth was found to be alive after sixty-four days.

On blood agar, however, the organism generally grows well and maintains its vitality and virulence for at least fifty days.[1]

To obtain cultivations from the human subject, the best method is to inoculate mice or rabbits with material, such as rusty sputum, containing cocci. The animal dies after a variable time, in the case of mice and rabbits, inoculated intraperitoneally in from about twenty-four to thirty-six hours, from pneumococcal septicæmia. From the blood drawn off in a sterilised pipette from the heart, through an area previously scarified, cultivations can readily be obtained (see p. 120).

Under the microscope, the organism is seen in the form of cocci, arranged singly, in pairs, in strings of four, and in long chains which are not unlike those of streptococci; but, on closer examination, the cocci forming the chain which may be arranged circularly, are seen in pairs, the pairs being separated by intervals greater than that which exists between the individuals of each pair.

The shape of the cocci in sputum, blood, or serous exudation, is generally distinctly lanceolate, each coccus being drawn out like the flame of a candle (fig. 61), the pointed extremities of each pair looking in opposite directions. This lance-shaped appearance is not always obvious in the cocci, and in cultivations—as a rule—is wholly lost, the organism being quite rounded or only slightly elongated. In the natural secretions and exudations, also, the pneumococcus is encapsuled, two or four cocci being surrounded by a quite homogeneous sheath or *capsule*, which has more the appearance of a 'halo' than a definite capsule; that is to say, the cocci are seen in the stained preparations to lie in small oval clear spaces, which are outlined by the counter-stain; e.g. eosin, if the Gram-Eosin method is employed. This halo-like appearance is lost in the case of cultures, the organism being then, for the most part, a distinct coccus; but where there is a collection of serum or fluid at the bottom of the two (as in the case of blood serum, &c.) encapsulated organisms may not infrequently be obtained, even from cultures. If a pure culture be injected into a susceptible animal, the rounded unencapsulated cocci are replaced in the serous exudation and blood of the animal by the more typical lanceolate, encapsuled organisms.

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[1] An easier and still more successful method is to completely fill the bulb of a large pipette (fig. 62) with blood infected with pneumococci. After sealing both ends of the bulb (leaving no air behind), it is kept in a dark cupboard until required for use.

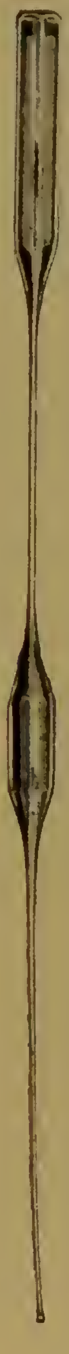
FIG. 62.—STERILISED GLASS PIPETTE, PLUGGED WITH WOOL AT THE UPPER END, OR MOUTHPIECE.
The free extremity of the capillary tube is fused in the blowpipe flame before sterilisation. Such sterilised pipettes should be kept ready for use in a sterile receptacle.

The pneumococcus is well stained by Gram's method, whereas the pneumo-bacillus of Friedländer is decolorised thereby.

Inoculation of animals.—Mice, rabbits, and guinea-pigs are very susceptible, especially the first two of these. The effects of inoculation have already been indicated, death occurring after the use of a very virulent culture in from twelve to twenty-four hours from even very minute doses; but it may not take place for several days, or perhaps even weeks,[1] if the sputum, or other secretions, exudations, &c., be used. Usually, however, a medium-sized guinea-pig, inoculated with a little rusty sputum, dies within forty-eight hours. After death, little or no change may be observed in the subcutaneous tissues into which the serum, &c., has been injected; but, in the peritoneal and pleural cavities, clear, serous, or blood-stained exudations are present, a variable amount of lymph being seen covering the peritoneum over the liver and great omentum. There may be no obvious naked-eye change in the appearance of the viscera. They may, however, be considerably congested, *e.g.* the lungs. On examination of the blood, and the exudations mentioned, the encapsuled organism may be seen frequently in large numbers.

Method of examining the heart's blood.—The heart being exposed aseptically,[2] a small area of its surface is scarified with a heated iron, or glass rod. The fused lower extremity of a sterilised pipette (fig. 62) is rapidly nipped off between finger and thumb, passed quickly through the Bunsen flame, and then pushed through the scarified heart wall into its interior, the heart being held steady with sterile forceps. Blood at once runs up the fine capillary tube, and may be sucked up so as to fill the bulb seen below the mouthpiece. The constriction of the tube above the bulb, and the wool plug in the mouthpiece, minimise the risk of infected material being drawn into the mouth. Cultures can be at once made by blowing a few drops of the blood, &c. into a tube of medium. Films may be prepared by letting a minute drop fall on to a clean coverslip held in cornet forceps. A second coverslip is applied to the first, and the two rapidly drawn apart, dried and fixed as usual. They may be stained with carbol-fuchsin, or Löffler's blue, or by the Gram-Eosin method.

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[1] For instance, when the sputum used, though still containing pneumococci, has been taken several weeks after the crisis has occurred, by which time the virulence of the organisms has diminished considerably.

[2] For further details see p. 152, *et seq.*

Immunity against the effects of virulent cultures has been successfully obtained in the case of susceptible animals, by previously inoculating them with attenuated, or with filtered cultures. The serum from such immunised animals is protective and curative for other animals. When mixed with a culture of pneumococci, the serum causes them to aggregate into clumps, as in Widal's sero-diagnosis of typhoid fever.

Friedländer's pneumo-bacillus.—This organism, to which, formerly, was ascribed a rôle at present credited to the pneumococcus of Talamon-Fränkel in the causation of croupous pneumonia, is now known to play only a secondary part in most cases. It occurs in the sputum and exudations in cases of pneumonia; in certain throat affections; and occasionally, also, in the saliva of healthy individuals. From its appearance in the earliest cultivations which were made, it was thought to be a coccus, and so it went by the name of *Friedländer's pneumococcus*, a term still used, rather un-

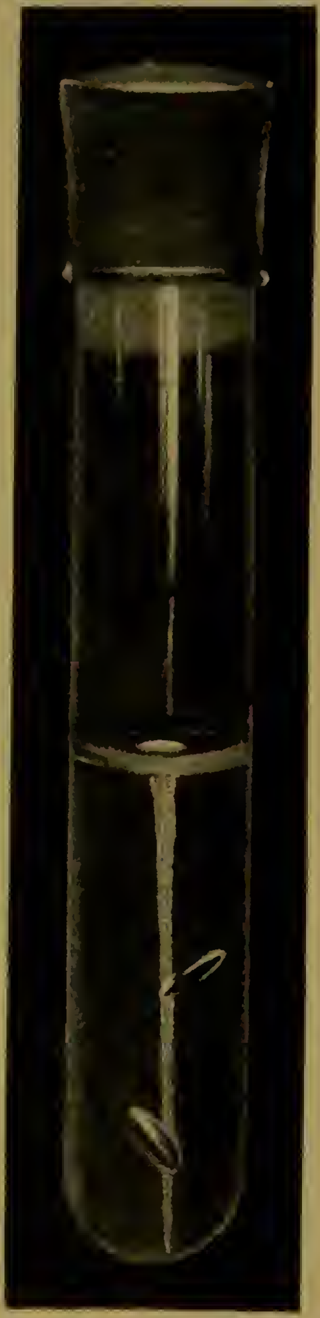

FIG. 63.—FRIEDLÄNDER'S PNEUMO-BACILLUS. GELATINE STAB-CULTURE SHOWING THE TYPICAL NAIL-HEAD APPEARANCE AND THE FORMATION OF GAS-BUBBLES, NOT ALWAYS PRESENT.

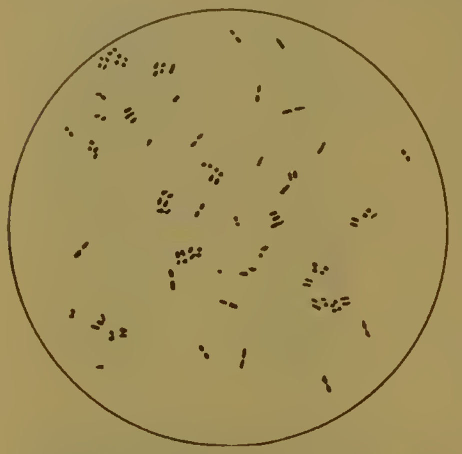

FIG. 64. FRIEDLÄNDER'S PNEUMO-BACILLUS. GELATINE CULTURE. ×925

fortunately, by some modern writers. There is no doubt, however, that this organism is a bacillus (fig. 64), though cocci-like forms may be seen in young cultures, associated with the short rods which make it up. It differs from the diplococcus pneumoniæ not only in its shape, but also by the fact of its being decolorised when treated by Gram's method, and still more notably by its comparatively exuberant growth on various media, such as nutrient gelatine, potato, &c.

Cultivations.—*Gelatine stab.*—The organism forms a typical 'nail' culture (fig. 63), the surface growth being excessive and raised into a moist, and white, or porcelainous knob, or 'head;' whilst the growth in the depth consists of a collection of white colonies grouped together into a column gradually tapering to a point at its lower extremity. Not infrequently, as in the illustration, gas bubbles are formed; but no liquefaction of the gelatine occurs. According to Crookshank, the nail-shaped cultivation is not always produced.

Gelatine streak.—A copious, moist, white, raised growth appears early on the surface, in striking contrast to what is seen in the case of the true pneumococcus.

Agar-agar streak.—A similar white, moist, growth is seen on the surface.

Potato, and blood serum.—Copious growths occur on both media, greyish-white in colour, in the case of serum; white and glistening, in the case of potato.

Broth quickly becomes turbid. *Milk* is coagulated.

Under the microscope, the organisms appear as cocci, and very short bacilli, to which the term *cocco-bacilli* might be applied in many cases, as they appear to be merely elongated cocci. Longer rods also occur. For the most part, the organisms are arranged singly, in twos and in fours, as in the case of the true pneumococcus; and, like it too, in the natural secretions, &c., the pneumo-bacillus is seen to be surrounded by a *capsule*, which appears in stained preparations as a clear space, or 'halo,' limited by a more or less sharply defined outline. In cultures, the same variety in form, varying from coccus to bacillus, may be seen, the capsule being generally lost, as in the case of the pneumococcus. From the exudation at the bottom of a serum tube encapsuled forms may be obtained, as also by inoculating animals subcutaneously, the resulting serous exudation being found to contain the bacilli in their encapsuled condition.

Inoculation experiments.—White mice are apparently the animals most susceptible to infection with serum cultures of this organism, death occurring in two or three days. Guinea-pigs are sometimes susceptible, and dogs still more so. Rabbits are immune.

Injection into the pleural cavity and into the lung in the case of mice is followed by a form of pleurisy, and pneumonia, respectively. Very numerous encapsuled organisms are found throughout the pleuritic effusion, and in the blood. The spleen is generally enlarged.

Micrococcus gonorrhœæ [1]

The gonococcus of Neisser has been definitely proved to be the specific cause of gonorrhœa, and by repeated experiment—though animals other than man have an immunity—this organism has been shown to comply with Koch's well-known postulates referred to on pp. 101–2.

The primary infection, in both sexes, appears to occur most frequently at some part of the urethra; in the male, at the lacuna magna, and at the bulbous and prostatic portions of the urethra, and the microscopic changes to be mentioned are also most marked at these three spots. Next to urethritis, the most frequent seat of primary infection in the female is the canal of the cervix, primary vaginitis being rare, the stratified epithelial lining apparently resisting invasion as successfully as the skin. A very persistent inflammation results from inoculation of the glands of Bartholin. In the male, suppuration may follow from infection of the follicles of the prostate, and very acute fatal pyæmia has resulted from septic thrombosis of the prostatic sinus, probably from a mixed infection. 'Prostatic threads' passed at the end of micturition, especially after compressing the prostate per rectum, may contain gonococci long after they have disappeared from any gleety discharge present. Hence the importance of examining the prostatic secretion, before deciding that a patient is free from infection. Swabbing out the urethra with silver nitrate so as to set up an acute urethritis, as practised by American surgeons especially, will often bring into evidence gonococci concealed in the follicles of the urethra long after the original discharge has ceased.

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[1] Realising the enormous importance of the subject, I have in this section summarised the recent, very exhaustive, investigations by Mr. Alexander G. R. Foulerton, F.R.C.S., recorded in his monograph, 'On the Micrococcus gonorrhœæ,' pp. 40–81, *Trans. Inst. Prevent. Med.* First Series, 1897.

Extra-genital gonorrhœal infection most commonly occurs at the conjunctiva, infants being infected at the time of delivery (*ophthalmia neonatorum*); and adults, by material conveyed from the urethra. Ulceration or sloughing of the cornea, ending in incurable blindness, may result, especially in neglected cases.

Mode of invasion.—The organisms appear to make their way through a breach in the superficial epithelium, in the case of the urethra. Cocci may also be seen lying between the cells lining the follicles of the mucous membrane. The deeper epithelium, and the leucocytes lying between them, and in the submucous tissue, are attacked by the cocci, which actively multiply in these cells, especially in the leucocytes, for which, compared with the few epithelium cells seen to be invaded (whether *in situ*, or loose in the discharge), they show a decided preference. They apparently do not get beyond the layer of dense plain muscular tissue limiting the mucous membrane; but the irritation caused by their presence in the submucous tissue results in a serous exudation, infiltrating the connective tissue, and a catarrhal condition of the mucous membrane, the epithelium becoming loosened and detached.

Secondary infection with other pyogenic organisms follows, purulent infiltration of the submucous tissue, with thrombosis of the capillaries, having been seen at this later stage. The gonococcus alone, however, has been found in some cases of pyosalpinx, and in other abscesses.

The exact nature of the chemical products of this organism is at present unknown, but they are responsible for the immediate effects of infection. In remote lesions, the gonococcus itself has been demonstrated, e.g. in gonorrhœal arthritis, endocarditis, salpingitis, &c. In a fatal case of gonorrhœal endocarditis, the specific organism has been found circulating in the blood during life.

Though definitely pyogenic, as in the instances quoted above, pure cultures of gonococci have been obtained from the joint in cases of gonorrhœal rheumatism without any sign of suppuration ever appearing.

Cultivation of the gonococcus cannot be carried out on any of the ordinary media of the laboratory, a most important point in distinguishing it from other diplococci which may be present in pus suspected to be of gonorrhœal origin. It has been shown that enough albuminous materials, in the form of pus, may be transferred to a tube of medium with the cocci for their immediate needs, though subsequent sub-cultures on to the same medium completely fail. As Foulerton insists, no medium can be considered as a suitable one until it has proved capable of bearing a transplantation of the coccus under conditions which will exclude any trace of the original pus. After careful trials, the same writer condemns, as useless, urine-peptone-broth, urine-agar, saline-peptone-agar, acid gelatine upon which, however (as well as on alkaline gelatine), a coccus which he has called the *Diplococcus urethræ communis*, larger than the gonococcus, and staining by Gram's method, grows well. Blood serum-agar, or agar to which other serous fluid such as serum from pleuritic effusion, ascitic, hydrocele or ovarian fluids, have been more or less successfully used. Mixtures of urine, agar, and serum, from human placental, and various other kinds of blood, have also been tried with varying success. Albuminous urine, from cases of nephritis, mixed with two parts of peptone beef broth, or with two parts of fluid agar, gives excellent results; as does a mixture of two parts of agar, kept fluid at 40° C., with one part of human urine, to which egg-albumin in the proportion of 5 per cent. by volume has been added.

The selected serum, diluted if desirable with a little fresh urine, is sterilised by aspiration through a Berkefeld filter, then mixed with the agar kept fluid at 40° C. in a water bath. Sloped tubes, or plates, (petri-dishes) may be prepared as usual. Plate cultures, made by lightly brushing some of the pus over three previously prepared plates, with the same camel-hair brush, in the way shortly to be mentioned, are absolutely essential in endeavouring to isolate the gonococcus from discharges (Foulerton).

An alternative way of making plates, recommended by the same author, is to take three tubes of sterile serum, inoculate the first with a loopful of pus, inoculate the second tube with one or two loopfuls from the first, and the third in like manner from the second. Add the contents of each tube to about twice the volume of agar, kept melted at 40° C., and pour into three petri-dishes as usual.

For general clinical work, Foulerton strongly recommends cultivation of the pus on 1½-inch agar plates streaked with fresh human blood, as first suggested by Abel.

The method of obtaining the blood aseptically from the finger-tip is to wash the skin with soap and water, then with 5 per cent carbolic acid, finally dipping the finger into alcohol and allowing it to drain off. A separate sterile pipette is used for collecting the drop of blood for each plate. With this capillary pipette Foulerton deposits a drop of freshly drawn blood from the finger on to the centre of each plate.

The smallest possible quantity of pus, if pus is to be inoculated, is then blown on to a small sterilised camel's-hair brush from the capillary tube in which it has been collected. This pus, with the drop of blood already deposited on the agar, is lightly rubbed over the surface of the first plate. The blood drop on the second plate is then rubbed out with the same brush, and so the blood on the third. This plan may also be adopted in cases of pyosalpinx, and gonorrhœal conjunctivitis.

In cases of gonorrhœal arthritis, the fluid aspirated should be mixed with two parts of agar, kept melted at 40° C. and plated.

Cultivations on serum-agar, or blood-streaked agar, are best kept between 35° C. and 37° C. Slightly raised, minute, sometimes almost imperceptible, moist, grey, or greyish-white colonies appear at the end of twenty to twenty-four hours, dotted over the surface of the plates. The colonies, being translucent, look like drops of dew when the plates are held up to the light. After seventy or eighty hours' incubation, when they may have attained a diameter of 1·5 to 2 mm., the colonies cease to grow. At first they appear, under the lower power of the microscope, to be rounded in outline, finely granular, of a faint brownish colour, rather deeper towards the centre. After seventy-two hours or so, the edge of the colony becomes irregularly rounded, and uneven at its margin. The darker, central, zone has also become more pronounced and opaque, and distinct collections of large granules may usually be seen. Foulerton notes the difficulty in removing young colonies from the medium with platinum wire, 'from the point of which it at once slips off again in a jelly-fish-like fashion.'

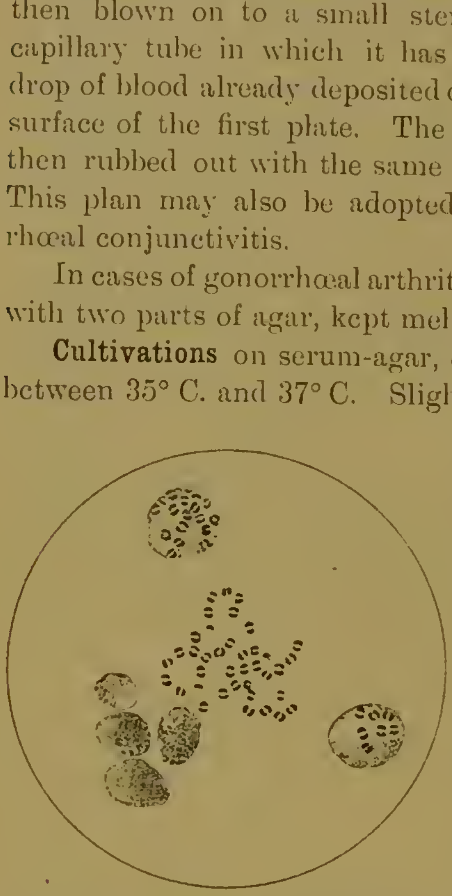

FIG. 65.—GONORRHŒAL PUS, WITH CHARACTERISTIC DIPLOCOCCI IN, AND BETWEEN, PUS CELLS. × 925

Inoculation experiments with cultures, or gonorrhœal pus, have only hitherto succeeded in man, not in animals.

Under the microscope, the gonococcus is seen (fig. 65) to be a coccus flattened, or slightly concave at one side, so as to be more or less kidney-shaped. The cocci are generally arranged in pairs, with the flattened, or concave, surfaces adjacent to each other.

Multiplication occurs by fission, the commencement of which is indicated by the concavity mentioned, the concave, or flattened, side being also that surface which was formed by the line of fission in the immediately preceding generation; i.e. the line of fission in each generation is at right angles to that of the previous generation (Foulerton). Hence there is a great tendency to the formation of tetrads and multiples thereof. The culture must be only lightly spread out in making the film preparation. The average measurements are said to be about ·7μ by ·5μ, though one coccus may be larger and retain the strain longer than its fellow when treated by Gram's method, by which rapid and complete decolorisation results for the film as a whole. Löffler's methylene blue is one of the best stains. Counter-staining with eosin, as directed in the exercise on p. 116, is very effective in the case of pus. The characteristic feature is the large number of pairs of kidney-shaped diplococci, which are present in the pus cells, and may even invade their nuclei. Paired diplococci are also seen lying loose, or grouped together in masses, between the pus cells. A minimum of four pairs of typical cocci in a single pus cell, is alone to be considered diagnostic, according to Foulerton.

A halo-like capsule may sometimes be visible, surrounding the diplococci in films from serum-agar cultures, but in pus its presence has been denied.

Besides the ordinary pyogenic cocci, eighteen different species of cocci have been isolated from the healthy, or diseased, male urethra; of these seventeen can be grown on agar at 36° C., or on gelatin at 20° C., the remaining species growing on agar, but not on gelatin. Subcultures on gelatin and agar from the plate colonies, prepared in the way above mentioned, should therefore always be made. If the organism grows under these circumstances it is certainly not the gonococcus.[1]

On the other hand, **the three special characteristics** which enable one to say positively that a suspected organism is the micrococcus of Neisser are—

(i) The occurrence in the pus cells of paired bean-shaped cocci—at least four pairs in each cell should be recognised.

(ii) The typical cocci should be readily decolorised when treated by Gram's method.

(iii) Pure growths cannot be subcultured on gelatine at 20° C., or on agar at either 20°, or 36° C.

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[1] For further details the student is referred to the monograph by Foulerton, *op. cit.* p. 55 *et seq.* Also Wurtz, *Bactériologie Clinique*, p. 299.

Micrococcus tetragonus

Micrococcus tetragonus, or tetragenus.—This organism was first isolated by Koch from the cavities in the lungs of phthisical patients, though—as shown by him—it has no apparent influence on the course of the disease in question. It is found in the walls of such cavities, as well as in the sputum, and also in non-tuberculous expectoration, and even in normal saliva. It has also been found in alveolar abscesses, and other forms of acute suppuration.

Cultivations.—*Gelatine stab.*—The growth along the track of the needle by the third day appears white, irregular, or granular. The individual granules, or colonies, enlarge, especially at the upper part of the medium, and at the surface ; a raised knob forms by about the end of the first week, looking like the head of a nail seen in profile. No liquefaction of the gelatine occurs.

Gelatine streak.—A slightly raised, creamy-white, growth is seen by the end of the second day.

Agar-agar, and serum.—Discrete, glistening, white colonies occur along the track of the needle.

Potato.—A moist, viscid, white growth is seen.

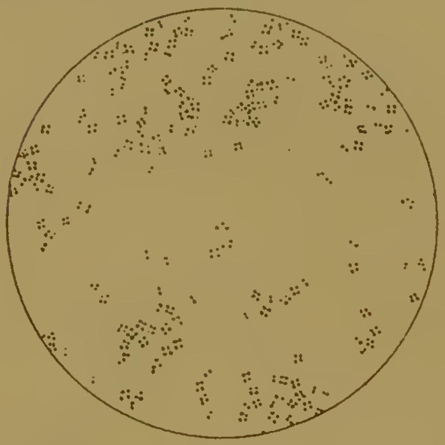

FIG. 66.—MICROCOCCUS TETRAGONUS. SERUM CULTURE. × 530

Under the microscope, the organisms appear as cocci, 1μ in diameter, arranged singly, in twos, and in fours. They are stained by Gram's method. There is a circular, gelatinous, halo-like capsule, containing cocci arranged as a tetrad. As a rule, however, the capsule is not seen in cultures.

Inoculation.—White mice, and to a less extent guinea-pigs, are exceedingly susceptible. A virulent culture produces a rapidly fatal result in from two to ten days, with a localised abscess at the point of inoculation, the organism being found in the capillaries of the organs, especially in the lungs, kidneys, and spleen, where they appear in, and between, the leucocytes (Crookshank). The corpuscles may be so crowded with the cocci as to present quite a granular appearance.

There are several species of the micrococcus tetragonus besides the one mentioned above.

A yellow variety of the *Micrococcus tetragonus subflavus*, similar in microscopic appearance to the foregoing, is found in the nasal cavity: not infrequently it is met with in the form of yellowish colonies, rapidly sinking into the medium, so as to lie at the bottom of a depression, in serum cultures made from cases of diphtheria, associated, of course, with the specific organism.

LESSON XV

ANTHRAX—TUBERCULOSIS—LEPROSY

A. **Bacillus anthracis**
B. **Bacillus tuberculosis**
C. **Bacillus lepræ**

A. Bacillus anthracis

I. *Cultivations*:
- (i) Gelatine stab.[1]
- (ii) Agar-agar, α. streak.
  - β. Three plates, prepared as usual (p. 25).
- (iv) Potato.
- (v) Broth.
- (vi) Broth, *containing* 0·1 p.c. phenol.

II. *Stain coverslip preparations* from *a culture*:
- (i) with carbol-fuchsin.
- (ii) by Gram's method.

III. *Stain blood*, and any other *exudation* present; also *films*, made by rubbing the coverslip against the *cut surface of spleen, liver*, &c., held in dissecting forceps, of an animal which has died from anthrax.

Stain (i) with Löffler's blue, or carbol-fuchsin.
(ii) by Gram-Eosin method.

IV. *Stain spores*, by Möller's method (p. 47) Use a culture which has been incubated at 37° C. for at least twenty-four hours.

V. Stain film preparations from the phenol-broth culture [I. (vi)] after a week's incubation. Use simple stains. No spores will be found. (*Asporogenous growth.*)

VI. *Stain sections of organs* from an animal inoculated with *B. anthracis*. Sections, imbedded in various ways, should be stained simply, and by the Gram-Eosin and the Eosin- (or Carmine-) Gram-Weigert methods (pp. 97–100).

VII. *Examine the agar plates*, after incubation, and make impression preparations, staining with carbol-fuchsin.

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[1] See first footnote, p. 35.

VIII. *Make a hanging-drop preparation*, but instead of using a broth culture, place a *drop of liquefied gelatine, or agar*, on the under aspect of a clean coverslip (held in cornet forceps), which has been sterilised by passing through the flame several times. Inoculate the medium, by means of a stab needle-point, with the smallest possible quantity of an anthrax culture, and place film side downwards over the hollow of the hollow-ground slide which has previously been 'ringed' with vaseline (p. 30). Watch the development of the spores in the bacilli as they are forming, and then that of the bacilli as they, in turn, are formed by the gradual elongation of the spores.

B. **Bacillus tuberculosis**

I. *Cultivations*: (i) Inoculate two serum tubes with a large loopful of curdy material from a freshly opened tuberculous abscess—*e.g.* of the hip-joint. Cap, and incubate at 37° C. for three weeks—examining daily. By the end of ten or twelve days, raised, opaque white, or creamy-white colonies will be visible. Subcultures may then be made on glycerine-agar.

(ii) Make subcultures on glycerine-agar from the pure culture given round. Use large flakes of the growth. Proceed as above.

II. *Stain coverslips*
of (*a*) Culture (i) with carbol-fuchsin; warm till it steams; after five minutes, wash very thoroughly.
(ii) by Gram's method.
(*β*) Phthisical sputum, by *Ziehl-Neelsen's method*.

Ziehl-Neelsen's method, for sputum

1. The sputum is placed in a petri, or other glass, dish, against a black background (such as a black vulcanite tray), and one of the minute, opaque, yellowish-white, caseous particles seen is transferred with sterile platinum loop to a clean coverslip held in cornet-forceps. Another coverslip—similarly held in forceps—is applied to the first, and by steadily rubbing, and then drawing the two coverslips apart several times, a uniformly thin film is left on each.

2. The films are dried, and then fixed, in the usual way, by passing them rapidly through the flame three times.

3. Carbol-fuchsin is filtered on to the coverslip, which is then held in the cornet-forceps and moved vertically up and down over the flame until vapour is seen to rise.[1] The stain remains on for five minutes afterwards, and the excess is removed by washing in water.

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[1] Where several films are to be stained, the cleanest and most rapid method (suggested to me by my friend Dr. W. G. Savage) is to *warm* (not boil) filtered

4. The coverslip is placed in 25 p.c. sulphuric acid [1] till the film *remains* almost completely decolorised on being placed in water. If a pink tint returns whilst in the water, dip once more into the acid, and then back into water, repeating this process till the pink finally disappears.

5. Dry the coverslip between the folds of a pad of German blotting-paper (p. 18).

6. Counterstain with Löffler's methylene blue, for two minutes.

7. Wash well, dry, and mount in Canada balsam.

The tubercle bacilli are red.

The sputum itself (and any organisms present besides tubercle bacilli) should be a beautiful *azure blue*.

This is the best way of demonstrating *B. tuberculosis* in sputum, in the urine, and in milk, &c., but in the case of the fluids mentioned, thorough centrifugalisation is necessary (see text, p. 143). Films are then made from the deposit.

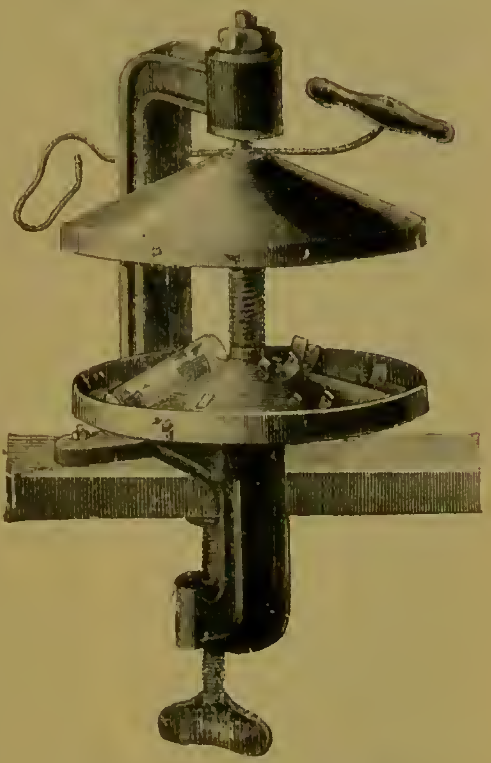

FIG. 67.—CENTRIFUGAL MACHINE, SPUN BY HAND, OR BY A WATER-MOTOR

If the sputum is very watery, it may also be centrifugalised, and the deposit stained in the above way.

Another method, which answers admirably for watery sputum,[2] is to add about 5 cub. cms. of the fluid to 100 cub. cms. of 5 p.c. carbolic acid in a sterile flask, which is then thoroughly shaken, and its contents poured into a sterile conical urine-glass. It is allowed to stand over-night, or for twenty-four hours, and the supernatant carbolic acid then poured away. Films made from the deposit are stained, as usual, by Ziehl-Neelsen's method.

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carbol-fuchsin in a sterile test-tube, and pour it on to the prepared films held in forceps. The subsequent procedure is as above.

[1] Some prefer to use 33 *per cent. nitric acid*.

[2] Crookshank has made the very important observation that the addition of an equal quantity of 5 per cent. solution of carbolic acid to phthisical sputum renders it innocuous in a few minutes, corrosive sublimate being unsuitable. (Hewlett's *Bacteriology*, p. 201.)

III. *Stain sections of organs* containing tubercle bacilli—e.g. phthisical lung.

It is essential to imbed tissues to be examined for tubercle bacilli, either in paraffin, or celloidin (see Appendix B, p. 273, for methods of imbedding, &c.).

Ziehl-Neelsen's method for sections of tissues containing tubercle, or leprosy bacilli

1. The section is fixed to the coverslip, and the paraffin completely removed (pp. 99–100).

2. The coverslip is held in cornet-forceps and carbol-fuchsin is filtered on—just as in the case of sputum (pp. 130–1, and footnote). The stain is warmed till vapour rises.[1] After ten minutes, the excess of stain is washed away and the coverslip placed, section side up, in a watchglass containing 25 p.c. sulphuric acid. It is left there undisturbed for twenty to thirty minutes (according to the thickness of the section) and then washed in water. If the colour returns, it is again placed in fresh acid for ten minutes or so, washed in water, and these processes repeated alternately till it is completely decolorised, or only the faintest pink tint left.

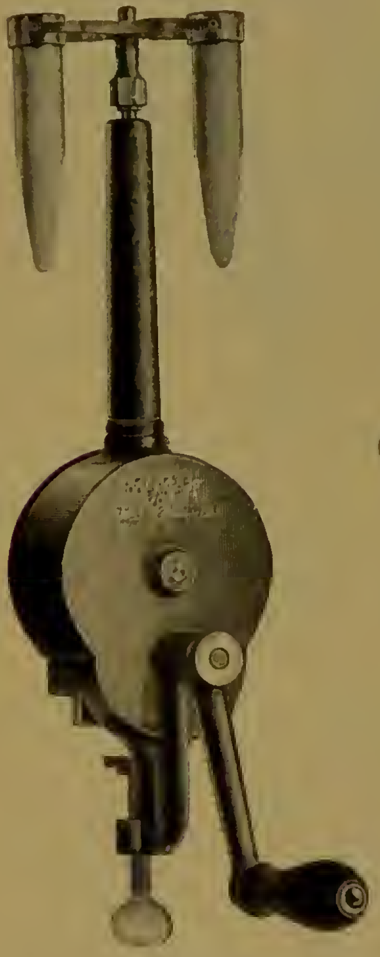

FIG. 68.—BECK'S HIGH-SPEED MEDICAL CENTRIFUGE

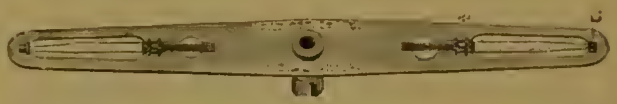

FIG. 69.—HÆMATOCRIT ATTACHMENT, OR FRAME, CARRYING TWO GRADUATED CAPILLARY TUBES FOR SEDIMENTING BLOOD, OR OTHER FLUIDS (TO TAKE THE PLACE OF THE ORDINARY TEST-TUBE CRADLES, SHOWN IN FIG. 68)

3. The tissue is counterstained in Löffler's blue for ten to thirty minutes.

4. It is then rapidly dehydrated[2] in absolute alcohol $\frac{1}{2}$ to 1

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[1] Sections may be placed, instead, for twenty-four hours, or over-night, in cold carbol-fuchsin, and then left for at least half an hour in 25 p.c. sulphuric acid.

[2] The dehydration with alcohol must be very rapidly performed, or most of the Löffler's blue will be dissolved out.

minute, and clarified[1] in xylol for two minutes.

5. Mount in xylol Canada balsam.

The tubercle bacilli are stained red, and the tissue, with any other organisms present, is stained blue.

C. Bacillus lepræ

Leprosy bacilli, occurring in the discharges from leprous ulcers, and in sections of tissues affected by the disease, may readily be demonstrated by Ziehl-Neelsen's method in exactly the same way as in the case of the *B. tuberculosis* (see text, p. 152, however, for differences).

Anthrax

Bacillus anthracis.—*Anthrax, malignant pustule, or wool-sorters' disease*, is caused by the Bacillus anthracis which, with its spores, is found in hides and furs, derived from foreign countries, by means of which those who have to prepare them become infected with the disease. The spores have also been found in atmospheric dust, and as the result of inhaling or swallowing such infected particles, internal anthrax may be established. The commoner mode of infection is through a wound or abrasion of the skin, and a simple pustule may become secondarily infected with the specific organism. There are thus two types of the disease, *internal or visceral*, and *external anthrax*. The exposed parts of the body are chiefly affected, a fact well brought out by W. Koch's analysis[2] of 1,077 cases, of which 905 occurred as follows: head and face 490, neck 45, upper limb 370.

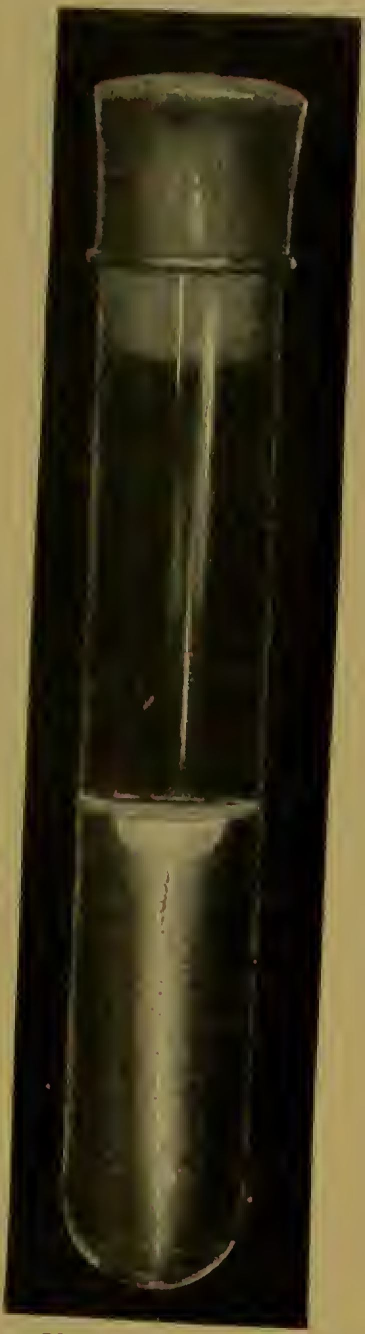

FIG. 70.—BACILLUS ANTHRACIS

Gelatine stab culture, showing characteristic growth, with commencing liquefaction, and cupping (from evaporation), at the surface of the medium.

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[1] Oil of cloves should rarely, or never, be used for clearing sections stained for bacteriological purposes, the anilin dyes being soluble in it.

[2] Treves's *System of Surgery*, vol. i. p. 318.

The disease in the skin commences as a dark papule, simulating the bite of an insect, upon which a vesicle full of clear serum soon arises. Subsequently new vesicles form a ring, encircling the now enlarged, deeply congested, almost black papule. This dark appearance, due to hæmorrhage, has suggested the French name of the disease, *charbon*. The English term *anthrax*, literally 'carbuncle,' was applied on account of the close resemblance of the two diseases at an early stage. In fact, even at the present day, especially in country districts, an ordinary carbuncle often goes by the name of 'anthrax'—a fact worth remembering. Infection spreads by the lymphatics, with great enlargement of the glands and œdema, which in the neighbourhood of the mouth or larynx may result in the most serious consequences.

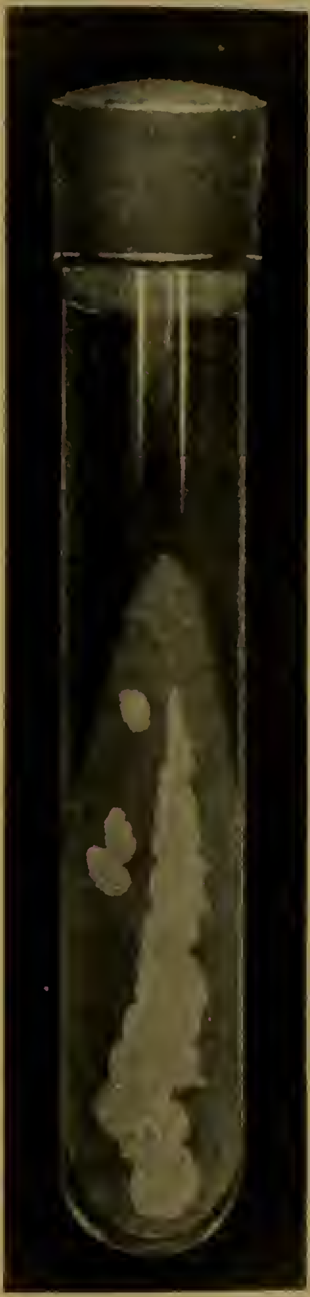

Fig. 71.—Bacillus anthracis
Glycerine agar-agar culture.

Cultivations.—*Gelatine stab.*—From the white growth which rapidly appears along the track of the needle, fine branches extend outwards into the surrounding medium, producing a very characteristic appearance (fig. 70), compared with that of an inverted fir, or 'Norfolk pine,' tree. Liquefaction of the gelatine occurs at the surface, and progresses fairly rapidly, so that by the end of about six days, the growth lies at the bottom of the tube as a flocculent white mass.

Agar-agar streak.—The growth is slightly moist, and of a dull, greyish-white appearance, especially near the margin. The surface growth is a little raised and the margins are somewhat indented, and much more translucent than the rest of the growth. On viewing this edge by transmitted light, its glistening appearance produces almost the effect of a fine mosaic. Isolated colonies may be seen. These are rounded or oval, and the character of the margin is as just mentioned. Colonies at the upper part of the tube, where the oblique surface of the medium is thinnest, can be examined under the low power of the microscope. The edge can then be made out to present the characteristic appearance described below.

Agar-agar plate cultivations.—Young colonies, not more than twenty-four hours old, are rounded or oval, sometimes indented at one end and pointed at the other, to form a heart-shape. When seen by the low power of the microscope, the surface is everywhere covered with fine lines, producing a striated appearance. The margin is often sharply defined at this stage. More advanced colonies, especially those on the surface, even in twenty-four hours may present the more characteristic appearance which has been generally compared with the 'Medusa-,' or 'Gorgon-locks,' the plaits of 'hair' being at first closely coiled and interwoven together; later, some of the 'tresses' get loose and spread far and wide to join other colonies in the neighbourhood. This appearance is perhaps better compared with the convolutions of the brain each convolution however, being

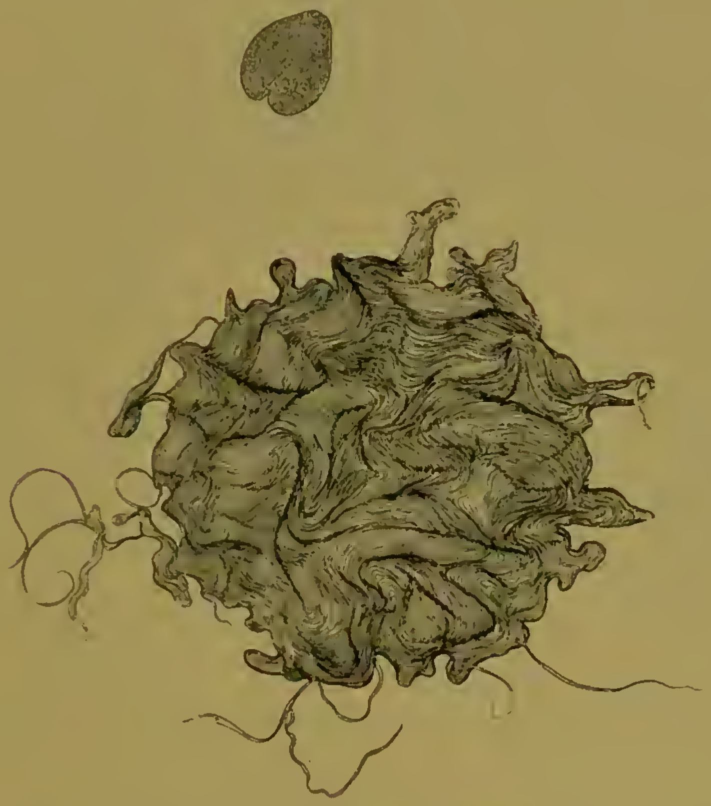

FIG. 72.—BACILLUS ANTHRACIS. × 50

Plate colonies on glycerine agar-agar. The smaller colony is about 24 hours old, and lay in the depths of the medium. The larger surface colony is a little older.

finely striated. Impression preparations can be made from these colonies (figs. 73 and 75).

Potato.—The copious growth, appearing after 24 to 48 hours, is opaque, creamy- or greyish-white, and somewhat dry. Spores readily form on this medium.

Broth.—Cloudiness sets in in a few hours, from the formation of little flocculent masses of growth, producing a very characteristic appearance. At the end of a few days, the medium becomes clear again, the growth having collected at the bottom of the tube as a flocculent white deposit. No scum is formed, contrasting with what is seen in broth cultures of *B. subtilis.*

Professor Sydney Martin has investigated the highly toxic, soluble products of the bacillus present in liquid cultures containing alkali-albumin, and shown them to be *albumoses*, causing fever, and an *alkaloid*, causing coma, when inoculated into animals.

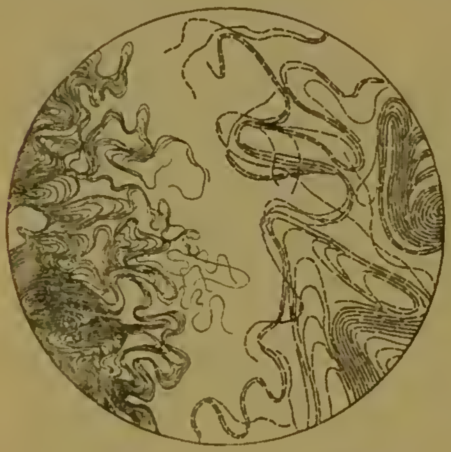

Fig. 73.—Bacillus anthracis
Impression preparation. Edge of the larger colony seen in fig. 72.

Under the microscope, the organism is seen to be rod-shaped, and to vary in length from 2 to 10μ (average 3 to 6μ), and to be about 1 to 1·5μ broad. Long threads are frequently found. Generally, the bacilli, which stain by Gram's method, appear to be square cut at their ends; but, frequently, these ends are seen to be a little broader than the rest of the organism, and instead of being flat they are slightly concave. This thickening and depression at the ends produces, in the case of a chain of bacilli, an appearance very like that of bamboo with its nodes. The depression or concavity at the end of the bacillus, just described, is, according to Symmers,[1] purely artificial, and results from the bacilli being overheated, prior to staining.

Spore formation is generally well marked, and can be actually seen in progress, by making a hanging-drop, employing, however, a loopful of liquid gelatine or agar-agar, and inoculating this, instead of using broth in the usual way. Spore formation is very rapid and can be observed to take place in such a hanging-drop in less than sixteen hours; and the development of the spore, by its gradual elongation into the bacillus, can also be readily made out.

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[1] Thoinot and Masselin's *Outlines of Bacteriology*, 1899, p. 106.

The hanging-drop, made from a broth culture, shows that the organism is non-motile (*B. subtilis* and *B. mycoides*, organisms resembling *B. anthracis*, are very motile).

Impression preparations, made from the agar-agar plates, and stained in the ordinary way, may be examined with the $\frac{1}{12}$ inch oil immersion-lens. The 'braided locks' are then seen to consist of innumerable bacilli with the above-mentioned characters, and the 'tresses,' spread out from the edge of the colony, are seen to consist of chains of these organisms.

Spore formation, even in such impressions, can readily be demonstrated by Möller's method (fig. 75); or, without special staining, can be made out in the form of highly refracting vacuole-like bodies (fig. 74).

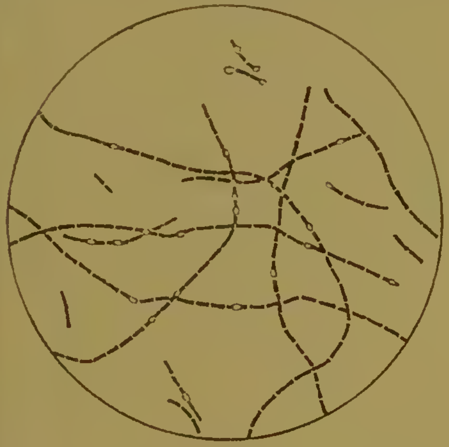
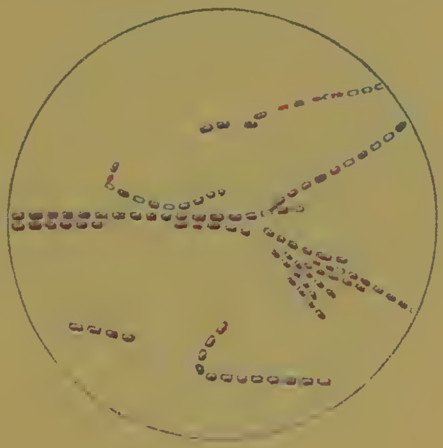


FIG. 74.—BACILLUS ANTHRACIS. × 925
The clear globular bodies are unstained spores.



× 925

FIG. 75.—SPORES OF BACILLUS ANTHRACIS
Stained by Möller's method. From an impression-preparation by the late Miss Mabel Webb, M.B.

As the culture gets older the sheath of the bacilli gives way, leaving the spores in chains still connected together by a small amount of the cementing substance. Then they separate from one another, and eventually give rise to young bacilli.

In blood freshly shed, and in the other exudations and tissues of the affected animal during life, no spores are seen, free oxygenation being a condition of their development. If the blood is exposed in a moist chamber for a fortnight or so, spore formation occurs.

Sporeless anthrax bacilli can be obtained by incubating a broth culture at 42° to 43° C., though refractile bodies (the *false spores* of Chauveau), or *microspores*, may be seen. By subculturing at 37° C., however, normal spore formation occurs.

Such **asporogenous growth**, with permanent loss of spore-forming capacity, can also be obtained by incubating, for eight to ten days, a phenol-broth culture, containing carbolic acid in varying proportions, from 1 : 2,500, up to 1 : 1,000 parts of broth.

Inoculation experiments.—Dogs, Algerian sheep, most birds, and the amphibia generally, are insusceptible to anthrax. Rats, also, are resistent to inoculation, but may become infected. Guinea-pigs, rabbits, white mice, the common English mouse, ordinary sheep, and cattle are highly susceptible; and it is probable that the Algerian sheep which have been found to be insusceptible have only become so by a process of natural selection.

Experimental inoculation indicates unmistakably that the disease is really a septicæmia, the organisms multiplying in the blood and tissues. *No spore formation*, as already stated, occurs in the body during life. There may be no change at the actual point of inoculation; but, starting from a spot close by, œdema, often blood-stained, is seen to spread throughout the subcutaneous tissues of the body. In this œdematous fluid the organism is readily demonstrated, and pure cultivations made therefrom. The muscles are pale; and, on opening the abdomen, little or no change may be obvious, though, as a rule, more or less blood-stained serous exudation, or even hæmorrhagic peritonitis, may be found. The spleen is enlarged and congested, the liver may be in a state of cloudy swelling, and the lungs present a congested appearance.

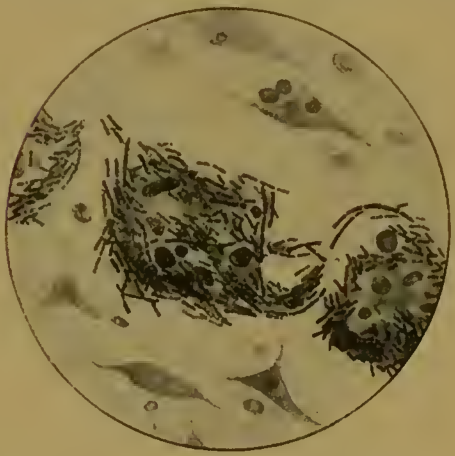


FIG. 76.—BACILLUS ANTHRACIS

Section of a cerebral cortex capillary (human), very faintly outlined, crowded with bacilli, which are escaping into the perivascular space and the tissue around.

But, frequently, however, no change is seen, even when the spleen and other organs are cut across with a sterile knife. When, however, a film preparation is made by drawing the coverslip, held in forceps, across the cut surface, drying, fixing, and staining with carbol-fuchsin, or Löffler's blue, in the usual way, myriads of bacilli are seen. The heart's blood is found teeming with organisms. Sections of all the organs show that the capillaries are universally invaded with masses of bacilli, and in sections of the brain (fig. 76) they may be seen passing through the capillary wall into the perivascular space, and invading the surrounding tissues. The absence of any definite lesion to the naked eye should be emphasised, as the writer has known of a case of a girl of fifteen in La Charité Hospital, at Berlin, who suffered from very obscure symptoms through life, so that a diagnosis could not be arrived at; and even at the *post-mortem* examination no definite cause of disease could be ascertained, until a coverslip preparation was made of the blood, when it was found to contain large numbers of anthrax bacilli, which were then found to be present in the liver and other organs.

The method of examining an animal supposed to have died from bacterial infection will be found at the conclusion of this chapter (p. 152).

Tuberculosis

The **Bacillus tuberculosis** is the organism which causes phthisis, and tuberculous disease generally. It is found invariably associated with these lesions, and from them it may be isolated in pure cultures, which, in turn, may be inoculated into susceptible animals, and the disease reproduced.

In the lesions in the inoculated animal the tubercle bacillus may be demonstrated, and pure cultivations again made; so that this organism conforms to all of Koch's requirements (p. 101), enabling one to affirm certainly that it is the cause, and not merely the concomitant, of tuberculosis.

Cultivations.—*Blood serum.*—In about twelve days, slightly raised, circular, or oval, white nodules are seen. They never become very copious, nor do they liquefy the medium. Blood serum is one of the best media for obtaining pure cultures from phthisical sputum, or from the foci of tuberculous disease in the tissues—e.g. in the case of a tuberculous hip-joint. After the white colonies mentioned above have appeared, they should be transferred to the glycerine agar, which is the more favourable medium.

Glycerine agar-agar.—Subcultures from blood serum thrive well on this medium, but it should not be employed for cultivations direct from a tuberculous lesion. After from twelve to sixteen days, small, opaque, rounded nodules are visible, spreading out from the seat of inoculation. In three weeks the extension of the growth is obvious. It is at first white and nodulated; later on, it becomes of a biscuit or light-buff colour. The surface becomes wrinkled or foliated, and a vigorous growth is not unlike the outside of a Stilton cheese. Less vigorous growths present a dry, slightly polished, shrivelled appearance with narrow, more or less horizontal laminæ standing out from the surface, reminding one somewhat of an agar culture of *B. subtilis*.

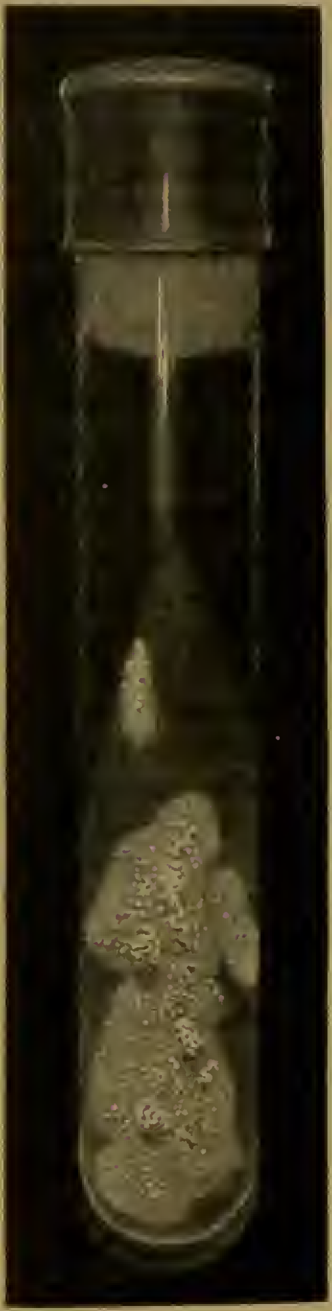


FIG. 77.—BACILLUS TUBERCULOSIS
Glycerine agar-agar culture, several months old.

Veal broth, to which 5 per cent. of glycerine has been added, has been recommended by Roux, and others, as a favourable medium. The growth appears as a white flocculent scum, which sinks to the bottom of the flask. If flakes are transferred to glycerine agar, typical growths are readily obtained.

Potato, if made alkaline with a solution of sodium carbonate, or by pouring over it nutrient broth, then capped, and kept at 37° C., shows white colonies in from two to three weeks.

Gelatine.—The organism will not grow on this medium. One practical reason why the organism does not grow on gelatine is that, when of the usual strength, it melts at 24° C., and the *B. tuberculosis* will not grow either below 29° C. or above 42° C.

Under the microscope, the organism appears as a slender bacillus, slightly curved; its length is from a quarter to half the diameter of a red corpuscle; but it varies considerably, so that its length has been variously estimated at from 1·5 to 5μ—the average being about $2{\cdot}5\mu$, its width being from ·2 to $\cdot 3\mu$. The bacillus frequently appears to be broken up, its protoplasm being aggregated into little points, so as to give it a characteristic 'beaded' appearance; indeed, this appearance has been attributed[1] to the presence of spores, a subject which will be referred to subsequently.

In young cultures the bacilli are often shorter and thicker than those seen in phthisical sputum, &c., and they generally stain more uniformly, so that the beaded appearance is less marked. In older cultures, on the other hand, the bacilli may be considerably longer than in sputum, and they exhibit well-marked beading. One end may be clubbed; very rarely, and in very old cultures, one or more branches may be seen. This branched condition is, though still rare, more commonly seen in the case of fowl (or 'avian') tuberculosis. Hueppe and his pupil Fischel[1] have brought forward evidence, based on investigations as to the branching of the mammalian tuberculosis organism, which tends to show that 'the tubercle bacillus is the parasitic growth-form of a pleomorphic mould and is not a true bacterium at all. In respect to its morphology, it is closely related to the ray-fungus' (actinomyces), which they regard as an undoubted mould. Coppen Jones, in subsequently confirming these results, has made it appear likely that this mould possesses a kind of fructification resembling chlamydo-spore[2]-formation (Hueppe).

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[1] The beaded appearance has also been attributed merely to the methods of film-preparation.

These views have not as yet met with general acceptance, but they will have an important bearing on the still vexed question as to the occurrence of spore-formation in *B. tuberculosis.*

The tubercle bacillus has a special sheath, and on this account it resists simple methods of staining; but, when once stained, it retains the colour very firmly, even when strong decolorising agents, such as 25 per cent. sulphuric acid, or 33 per cent. nitric acid, are brought in contact with it. This character is made use of in the staining of phthisical sputum, and tuberculous tissues generally. These are first stained with a warm solution of carbol-fuchsin, a decolorising agent is then applied, so that the sputum, or tissues, to the naked eye appear completely decolorised. Under the microscope, however, at this stage the tubercle bacilli would be seen to be stained red, all other structures being decolorised. It is convenient to counterstain the latter with Löffler's methylene-blue, so that the red tubercle bacilli are thrown up in relief against the blue background, which consists of the tissues, or cells, pus, &c., and of any organisms which may be present other than tubercle bacilli. This is Ziehl-Neelsen's method (for details, see p. 130); and, although the tubercle bacillus takes

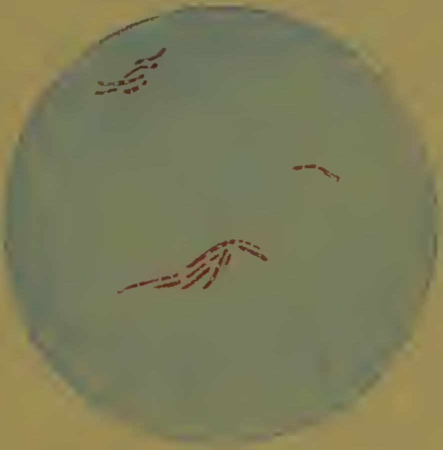

FIG. 78.—TUBERCLE BACILLI IN CENTRIFUGALISED DEPOSIT. × 925

From a case of urinary tuberculosis. Stained by Ziehl-Neelsen's method. (See also fig. 79.)

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[1] Hueppe, *The Principles of Bacteriology*, p. 42 (figures given).

[2] See p. 92.

Gram's method of staining, practically Ziehl-Neelsen's is the only method used.

Spore-formation in the case of *B. tuberculosis*, though highly probable, has not yet been proved satisfactorily. The beaded appearance of the bacilli so commonly seen, especially obvious on staining by Gram's method, and the clear vacuole-like spaces intervening between the aggregated masses of protoplasm (more noticeable in some specimens than in others), have been attributed to the presence of spores. The statement that such beads have been demonstrated to be spores, by staining with Möller's method, remains unconfirmed. On the other hand, the fact that these beads take the simple stains, carbol-fuchsin, &c., just like the rest of the body of the bacillus, is opposed to the belief that they are spores. Chlamydo-spores are described by those who regard this organism to be a mould and not a true bacillus (p. 141).

In this connection, the following facts are of importance :

Cultures of B. tuberculosis are killed by heating for ten minutes to 70 C., according to Yersin, who has shown that a solution of 1 in 20 carbolic acid causes death in thirty seconds.

Phthisical sputum is rendered innocuous by momentary heating to 67°–68° C., according to Macfadyen and Hewlett,[1] 'so that milk which has been "**pasteurised,**" *i.e.* heated to 68°–70° C. for twenty to thirty minutes, may be regarded as quite safe.' See also a footnote on p. 131.

Sections of tuberculous tissues, which should have been previously imbedded in paraffin or celloidin, are best stained according to Ziehl-Neelsen's method ; but unless dehydration by absolute alcohol is very rapidly performed, counterstaining of the tissue and organisms other than tubercle bacilli, by methylene blue, is difficult.

In caseating lymphatic glands, pus, very chronic tuberculous lesions generally, tuberculous synovial membrane, salpingitis, and lupus, the demonstration of tubercle bacilli is not always easy. A series of sections should be examined.

As it may prove to be a serviceable method, it may be mentioned, however, that the writer has found as many as a hundred tubercle bacilli in a caseating gland, from a case of senile tuberculosis, on staining a film obtained by rubbing a coverslip over the cut surface.

Of the histological changes, little need here be said. Besides, or in place of, the formation of typical giant-cell systems—which, however, are not absolutely pathognomonic for tuberculosis—there may be merely the production of granulomatous tissue.

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[1] Hewlett, *Manual of Bacteriology*.

The section should, therefore, be first examined under the two-third inch objective for giant-cells, with peripherally placed nuclei. If these are present, tubercle bacilli are carefully sought for with the oil-immersions lens, within and around the giant-cells.

In the absence of these large cells, or if no bacilli have been found in them, prolonged examination should be made of the aggregations of smaller round cells, resembling granulomatous tissue, present in the section.

Such tissue may be really teeming with tubercle bacilli, even in the entire absence of giant-cells, as in a case of tuberculous meningitis examined by the author.

Tuberculous milk, and urine,—in cases of supposed tuberculosis of the urinary tract generally,—are examined in exactly the same way as sputum, after centrifugalising them by means of one of the machines figured on. pp. 131–2. The milk, or urine, is placed in the glass tubes provided, these having been previously sterilised with every precaution. The method recommended is to clean away obvious dirt with a test-tube brush, then soak the tube for at least ten minutes in concentrated sulphuric, or nitric acid; the acid is poured off and the tube rinsed in tap-water, then in distilled water, and finally in rectified spirit. This is allowed to drain away, and the tube is plugged with a sterile rubber stopper. No more than an inch, or an inch and a quarter, of fluid is put in the tube, as when placed in the machine it occupies nearly a horizontal position. The older pattern of centrifugal machine (fig. 67) is worked by hand, but it may be adapted to a water-motor, which is a much more convenient method. All milk, &c. must be centrifugalised before a negative decision is come to as to the presence of tubercle bacilli; even a repeated examination may sometimes fail to reveal their presence, which can then only be ascertained positively by inoculating susceptible animals, such as the guinea-pig.

The carbolic acid method (p. 131), which has been employed successfully for the detection of tubercle bacilli in watery sputum, might, in an emergency, also be tried for urine, &c., should the centrifugal machine not be available.

Certain precautions should always be adopted in thus investigating cases of urinary tuberculosis. The urine should be passed straight into a carefully sterilised bottle fitted with a new cork, either through a sterile glass funnel, or drawn off with a previously boiled Jacques's catheter. A glass catheter may be used in the case of women. The orifice of the urethra and the adjacent parts should first be carefully cleansed. These precautions are necessary because fallacies may result both from the employment of urine glasses in constant use in the wards of a hospital, and from the presence of the **Smegma bacillus**, present in the caseous-looking secretion collecting between the prepuce and the glans penis, between the scrotum and thigh, between the labial folds, &c.

The *smegma bacillus*, *Lustgarten's syphilis bacillus*, and the *tubercle bacillus* closely resemble one another in size and general appearance, as well as, but to a less extent, in the difficulty with which they take up a simple stain—e.g. carbol-fuchsin—and the tenacity with which they hold that stain, when treated by Ziehl-Neelsen's method.[1]

So much stress has recently been laid upon these resemblances that it has been thought necessary to allude to the differential diagnosis.

The differences in staining reaction are as follows:

The *smegma bacillus*, when stained by Ziehl-Neelsen's method, retains the carbol-fuchsin dye, even after treatment with 25 per cent. sulphuric acid, unless exposure to the acid is considerably prolonged, but is rapidly decolorised by alcohol. Neither the smegma nor the syphilis bacillus is pathogenic for animals.

The *Syphilis bacillus* of Lustgarten is at once decolorised by 25 per cent. sulphuric acid, but is less susceptible to the action of alcohol. Confirmation of Lustgarten's claim to have discovered the organism of syphilis is not yet forthcoming, whilst some have even thought the syphilis and smegma bacilli to be identical.[2]

The retention of the stain after exposure to acid in the case of the smegma bacillus appears to depend on some fatty body present in the sheath of the organism. This may be removed by placing an unstained film in absolute alcohol for three hours, and then in chromic acid for fifteen minutes. Subsequent attempts at staining by Ziehl-Neelsen's method will result in the decolorisation of smegma bacilli by the acid, so that they finally appear blue from the methylene-blue counterstain, any tubercle bacilli present remaining of a red colour.

The precaution of drawing off the urine by catheter, after cleansing the meatus, greatly minimises the possibility of contamination with smegma bacilli.

It has been stated by French writers that in two-thirds of the cases of undoubted urinary tuberculosis, the tubercle bacillus is not to be detected by microscopic examination alone, English writers stating this proportion to be one-half, the positive results in the other cases only being obtained by the inoculation of susceptible animals.

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[1] The similarity between tubercle and leprosy bacilli is dealt with subsequently.

[2] The already-mentioned differences in staining reactions are, however, opposed to this view.

After examining a very considerable number of urines from cases of supposed urinary tuberculosis occurring in hospital and private practice, personal experience leads me to the belief that prolonged examination of the stained, centrifugalised, urinary deposit by any careful observer will, in most cases, give a positive result, if the case be really one of tuberculosis. In the event of a negative result, where the clinical features of the case favour the diagnosis of tuberculosis, inoculation of highly susceptible animals, such as the guinea-pig, should be resorted to, as this is the most reliable method of all, a reliable, positive result being obtainable in a fortnight, or even earlier (see p. 148).

The inoculation of animals for diagnostic purposes, with a fluid suspected of containing tubercle bacilli.

The most suitable animal is a guinea-pig, and the method recommended is to inject into the subcutaneous tissue in the neighbourhood of the left knee joint, 1 or 2 cub. cms. of an emulsion made by rubbing up in sterile nutrient broth, or normal saline solution, the suspected material, sputum, centrifugalised urinary deposit, &c.

Two animals are used: the first is killed in a fortnight's time, and if the lesion, to be mentioned, is found, the second animal is killed in three weeks' time; but should there be no evidence of tuberculosis in the first animal, the second is kept for four or five weeks. Film preparations to show the tubercle bacilli may be made from the caseating lymphatic glands, as also pure cultures on serum.

The advantages of this method of inoculating guinea-pigs for the diagnosis of tuberculosis were pointed out by Sheridan Delépine.[1] The accompanying drawing (fig. 79) has been made to illustrate the effect of such inoculation with the centrifugalised deposit from the urine from a case of tuberculous disease of the kidney. The centrifugalised deposit itself, from the same case, shows (fig. 78) the presence of tubercle bacilli in clumps.

Lesions in the inoculated animal.—As Sheridan Delépine pointed out (*loc. cit.*) the lymphatic glands are affected in a definite order by the tubercle bacilli injected, so that, beginning from the seat of inoculation in the region of the left knee joint, the first one to be affected is the left popliteal gland. This shows well-marked caseation, and is frequently reduced to a mere bag of 'pus,' which may escape into the tissues, forming an abscess between the muscles of the thigh, extending almost up to the pelvis.

The next glands to be affected are, in order, the left superficial

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[1] *Brit. Med. Journ.*, September 23, 1893.

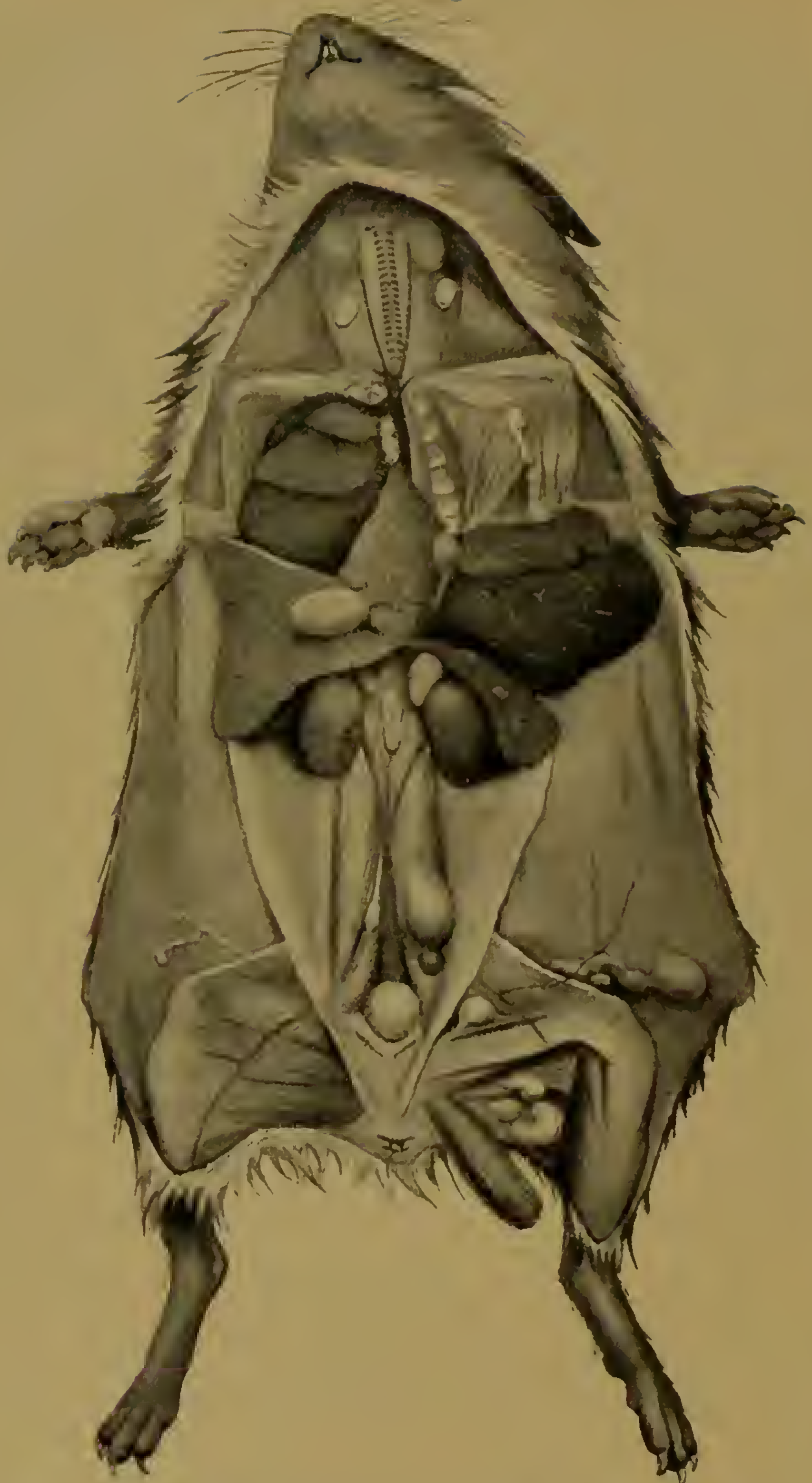

FIG. 79.—EXPERIMENTAL TUBERCULOSIS IN A GUINEA-PIG (ABOUT THE THIRD WEEK AFTER INOCULATION)

This animal was injected by the author, according to the method advocated by Sheridan Delepine, with the centrifugalised deposit from urine, from a case of tuberculous pyelitis. The coverslip preparation, fig. 78, was made from the centrifugalised deposit from same case.

and deep inguinal, the sub-lumbar (adjacent to the left common iliac vessels), the renal, and a gland in the hilum of the liver, which can only be well seen on turning aside the stomach and pushing up the liver, so as to fully expose its under surface. This gland lies close to the head of the pancreas. Nodules of disease are also frequently observed in the spleen (fig. 79).

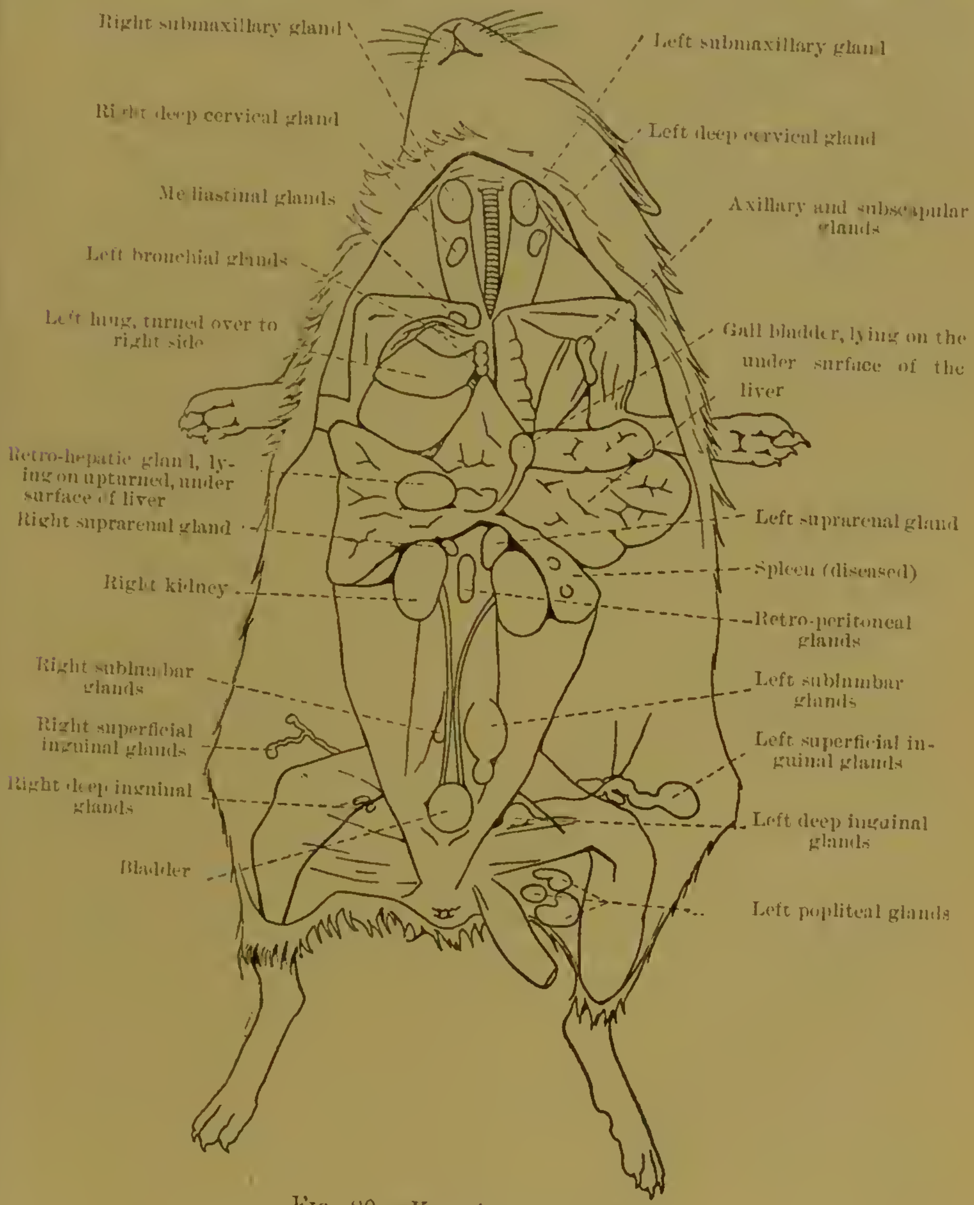
Right submaxillary gland
Left submaxillary gland
Right deep cervical gland
Left deep cervical gland
Mediastinal glands
Axillary and subscapular glands
Left bronchial glands
Left lung, turned over to right side
Gall bladder, lying on the under surface of the liver
Retro-hepatic gland, lying on upturned, under surface of liver
Right suprarenal gland
Left suprarenal gland
Spleen (diseased)
Right kidney
Retro-peritoneal glands
Right sublumbar glands
Left sublumbar glands
Right superficial inguinal glands
Left superficial inguinal glands
Right deep inguinal glands
Left deep inguinal glands
Bladder
Left popliteal glands

FIG. 80.—Key-plan to fig. 79

Passing through the diaphragm, the tubercle bacilli affect the glands around the root of the left lung and in the axilla of the same side, then pass upward into the glands of the neck. All this infection takes place before the lymphatic glands of the opposite side of the body become affected, and the appearance presented, even so early as the second or third week, is very striking. The diseased (tuberculous) left sublumbar gland may, for instance, be an inch long, by quarter of an inch wide, whilst the unaffected right gland—often less than half an inch away on the other side of the body—may be so small as to be only just perceptible (fig. 79).

Sheridan Delépine[1] states that:

> Inoculation of the skin on the inner aspect of the left hind limb, just below the knee, gave the following results:
>
> (*a*) Before the end of the second week there was marked tuberculosis of all the lymphatics below the diaphragm, on the side inoculated. The spleen was distinctly affected. The liver was beginning to be involved.
>
> (*b*) Before the end of the third week, in addition to the above organs, the liver was distinctly, the lungs were beginning to be, and the bronchial and mediastinal glands were distinctly affected.
>
> (*c*) At the end of the fourth week, and up to the end of the fifth, the glands of both fore limbs, and, to a less extent, of the neck, began to be involved on both sides of the body.
>
> (*d*) Before the end of the seventh week, the superficial inguinal glands, on the side of the body not inoculated, began to enlarge.
>
> (*e*) Before the end of the eighth week, the deep inguinal glands, on the side not inoculated, began to enlarge.
>
> (*f*) Even at the end of fifteen weeks, the sublumbar gland on the right side showed little evidence of tuberculosis, and the popliteal none.

Tuberculosis in animals.—This disease, whilst very commonly occurring in oxen and fowls (**avian tuberculosis**), is rare in horses, cats, dogs, and pigs, and very rare in sheep and goats.

Though the lungs, pleura,[2] lymphatic glands, and liver are generally most affected in cows, tuberculous mammitis is always the chief lesion to be feared, as otherwise the milk and flesh appear not to contain the specific organism, or to convey the disease, to judge by Nocard's experiments. In fowls, the abdominal viscera are chiefly attacked.

The bacillus in human and bovine tuberculosis is identical. Some doubt exists in the case of avian tuberculosis, partly on account of the fact that its cultures flourish vigorously at 43° C., whilst those of human tubercle bacilli cease to grow at 41° C. Further, dogs are susceptible to human, but insusceptible to avian tuberculosis, whilst fowls, highly susceptible to avian, are more or less insusceptible to human tubercle (Strauss and Gamalcia).[1] In spite of these facts, and the dryness and scaliness of cultures of the human variety, compared with the moist, greasy look of avian cultures, further investigations seem to lead to the conclusion that the two bacilli are not distinct species, but races of the same organism.

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[1] Sheridan Delépine, 'On the Value of Experimental Tuberculosis in Diagnosis,' *Brit. Med. Journal*, pp. 665-6, vol. ii. 1893. Important details are given in 'The Spread of Tuberculosis through the Lymphatics,' *Med. Chronicle*, May 1894.

[2] When affecting the pleura, pendulous masses of nodules are formed on the surface, the disease being then known by the name of 'grapes,' a figure of which will be found in Crookshank's *Bacteriology*.

Tuberculin.—Koch's original tuberculin is prepared as follows. Glycerin-veal broth cultures, which need not be virulent, are grown for from six to twelve weeks in large shallow flasks, so as to facilitate free oxygenation and the formation of a copious surface growth. These liquid cultures are concentrated to one-tenth of their original bulk, and are then passed through a Chamberland filter.

The filtrate, viscid on account of the glycerin present, and of brown colour, produces no effect when injected into healthy animals, or human beings; but extremely minute doses, where tuberculosis, or lupus, is present, cause a sharp, though temporary, elevation of temperature, more or less collapse, and the formation of localised swellings due to acute hyperæmia, with great distension of the capillaries in the areas where the disease is located.

In lupus, especially, necrotic changes occur with much exfoliation; after repeated doses marked improvement is apparent for a time. In most cases there is recurrence in the healed areas.

This reaction to tuberculin is now extensively made use of as the best mode of detecting latent tuberculosis in cattle.

Later tuberculin preparations.—In 1897 Koch introduced three new tuberculins,[2] distinguished by the letters T A, T O, and T R, signifying, respectively, *alkaline*, *upper* (Germ. *ober*), and *residual tuberculins*. For their manufacture, young, and very virulent, cultures are necessary.

Tuberculin A, or T A, is extracted from such cultures by means of a 10 per cent. solution of caustic soda, and the solution is filtered. The fatal objection to its clinical use is the formation of abscesses, which do not result with the use of T O, or T R.

Tuberculins O, and R, (T O, and T R), are prepared by vigorously triturating (pounding) in a mortar, driven by machinery, dried cultures of tubercle bacilli, and then adding distilled water. The emulsion is thoroughly centrifugalised. The supernatant clear, but opalescent, fluid in the upper part of the tube is free from bacilli, and constitutes in the case of this first centrifugalisation tuberculin O, (T O).

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[1] Quoted in the English edition of Thoinot and Masselin's *Outlines of Bacteriology*. As a matter of fact, as the editor states, fowls have been successfully inoculated with human tuberculosis.

[2] See *Deut. Med. Woch.*, April 1, 1897; or *Brit. Med. Journ.*, April 7 *et seq.*, 1897.

The *débris* of tubercle bacilli at the bottom of the tube is used for the production of tuberculin R, (T R). It is dried, triturated with distilled water, and centrifugalised again and again, till hardly any *débris* remains. The several portions of supernatant fluid removed in these repeated operations all act similarly, and are classed together as tuberculin R, (T R).

To preserve T O and T R, 20 per cent. of glycerin should be added.

It should be said that T R is alone used clinically. The liquid contains 10 milligrammes of solid matter per cub. cm., the dose being slowly increased from $\frac{1}{500}$ to 20 milligrammes of the solid matter (Hewlett).

The most remarkable property of the new tuberculin (but this is chiefly confined to T R) is that by its previous use, in graduated doses, complete immunity can be conferred on such highly susceptible animals as guinea-pigs, experimentally inoculated with virulent cultures of tubercle bacilli. Whatever may be said of the results of its clinical use, there can be no question as to the immense scientific value of this discovery of the immunising properties of T R, which are undoubted.

Pseudo-tuberculosis is the name which has, rather unfortunately, been applied to certain diseases in which, to the naked eye, the morbid appearances resemble to a greater or less degree those of true tuberculosis. A variety of micro-organisms, strepto-thrix filaments, and moulds, e.g. *Aspergillus fumigatus*, have been found in these lesions. A very common parasitic worm in sheep, the *Strongylus filariæ*, produces small pearly-grey glistening nodules in the lungs, and these have frequently been mistaken for tuberculous disease.

In a recent discussion[1] on this subject, the Committee of the Pathological Society of London rightly condemned the further use of such a misleading and unscientific term as pseudo-tuberculosis. The word 'tuberculosis' ought to be strictly limited to disease ascertained to be due to tubercle bacilli; and, wherever possible, the less ambiguous terms 'nodule,' 'nodular' should replace the words 'tubercle' and 'tubercular.' The systematic bacteriological examination of cases in the post-mortem room would do away with the necessity for using terms convenient clinically, perhaps, but rather loose and misleading otherwise.

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[1] *Trans. Path. Soc. Lond.* 1899.

Leprosy

This disease, known to the ancients under the name of Elephantiasis Græcorum, attacks primarily either the skin or the nerves; secondarily, other tissues become involved. Two types of disease are thus commonly described: (1) the skin, tubercular, or nodulated, leprosy; (2) the nerve, or anæsthetic, form; a third, but less common, type is the mixed, or complete, leprosy. The anæsthetic form is said to be more frequent in tropical countries, and the nodular in Europe.

B. lepræ.—The organism of leprosy presents a striking resemblance to that of tuberculosis, both microscopically, and as regards its staining reactions. It is rather thinner, but, generally speaking, a little longer (5 to 6μ), than the tubercle bacillus (2 to 4 or 5μ), though considerable variations occur. The diseased areas of skin and nerves are often crowded with bacilli (fig. 82), generally situated within large, ovoid, 'lepra cells.' They present a typical appearance, the protoplasm being aggregated into a series of little beads, separated by clear vacuole-like spaces. Some stain uniformly, and of those seen in the discharge from an ulcer (fig. 81) a good many are decidedly smaller than the average tubercle bacillus. The beaded appearance in the case of both organisms mentioned has led some to believe that it may indicate spore formation, but this is extremely doubtful (see p. 144).

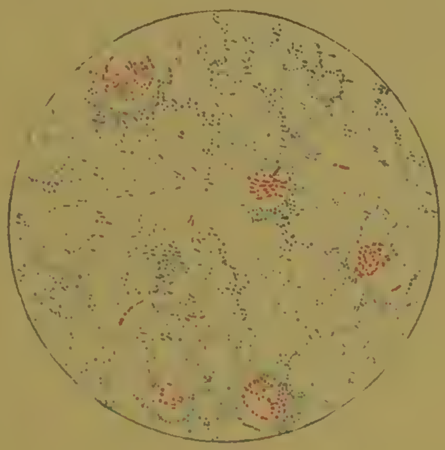

FIG. 81.—B. LEPRÆ IN DISCHARGE FROM ULCER ON THE GREAT TOE. × 925
Notice the aggregation into ovoid areas, probably degenerated lepra cells. (Ziehl-Neelsen's method.)

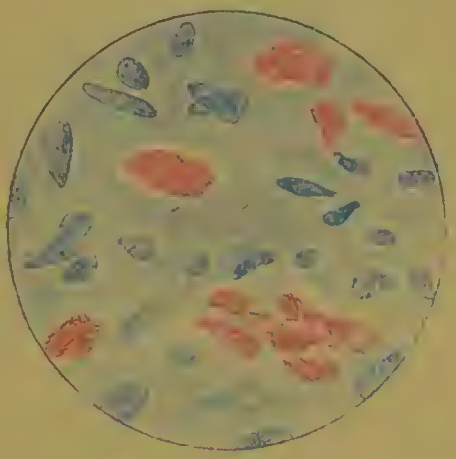

FIG. 82.—B. LEPRÆ IN LEPRA CELLS IN SKIN
(Stained by Ziehl-Neelsen's method.)

The leprosy bacillus readily takes the simple stains. The beaded appearance is well brought out by Gram's method. Ziehl-Neelsen's method, however, is generally employed, exactly as in the case of B. tuberculosis.

Though the discharge from the ulcers and the diseased tissues contains the organism in such large numbers, cultivation outside the body has, so far, not been very successful. Campana, of Rome, claims to have done so in stab glucose agar, but complete proof that the organism thus isolated is that of leprosy is not yet forthcoming.

In sections, leprosy bacilli occur aggregated together in large numbers, chiefly in the ovoid lepra cells to which reference has already been made. The appearance thus presented strongly contrasts with that observed in sections of tuberculous tissue.

In the discharge from leprous ulcers, the same tendency to aggregation into ovoid masses is often noticeable, and this is probably due to the bacilli being within a lepra cell, the outline of which, however, is often indistinct, or invisible. Discharges from such patients may, therefore, prove to be a serious source of contagion.

Contrasting the staining reactions of the two organisms, it may be stated that the leprosy bacillus takes the stain quicker, even in the cold, and retains it more firmly even on exposure to 25 per cent. sulphuric acid, than in the case of the tubercle bacillus, which stains better in the warm than in the cold.

Method of examining an animal dying of an infective disease.—It will be convenient here to mention the best way of making a *post-mortem* examination of an animal whose death is suspected to be due to micro-organisms. Three pairs of scissors (stout, medium, and fine), three sets of scalpels, and three pairs of dissecting forceps are sterilised by boiling.[1] An enamelled frying pan is very useful for this purpose. The animal being dead, or, if necessary, killed by chloroform, is pinned out on a board, the abdominal surface upwards. A bowl of 1 in 1,000 perchloride of mercury lotion is always to be kept at hand, partly in case of accidents. With this the hair, which may be previously clipped short, is thoroughly douched, and by means of the scalpel (or scissors) and forceps the skin is reflected in the median line in its whole length from the hyoid cartilage downwards. It is turned completely aside, so that none of the fur shall get in the way during the subsequent incisions. Before incising the muscular layers, lymphatic glands, where exposed, should be examined to see if there is any evidence of tuberculosis. The chief glands are the superficial, and deep, inguinal, and the popliteal (see diagram, fig. 80).

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[1] As the use of boiling water (as also a solution of 1 in 20 carbolic acid) destroys the sharp edges of knives, and tends to rust hypodermic needles, boiling oil is recommended, especially for hypodermic needles, at the Pasteur Institute at Paris.

The abdominal muscles are then swabbed with the perchloride lotion; or, in the lines of the incisions that are to be made, a bar of iron, or glass rod, heated to redness may be laid. This forms white linear scars, the tissues of which are thus rendered sterile; incisions may now be safely made along these lines, using the second set of sterile instruments. It is convenient to make a median incision from the extremity of the sternum to the pubes, and also to cut obliquely along the costal margins. Incisions are then made at right angles to the median one, about the level of the groin on either side, so that two large flaps can be turned completely aside, exposing the contents of the abdomen. Should there be any serous exudation, or hæmorrhage, or lymph, some of it is sucked up in sterile pipettes (fig. 62), and inoculations made at once by blowing some of the contents into tubes of suitable media. Coverslip preparations may be made at the same time, for which simple staining with carbol-fuchsin, or Löffler's blue, is generally all that is necessary, though the Gram-Eosin method is often very effective.

The intestines, if showing no obvious disease, are turned aside, using the third set of sterile instruments. The lymphatic glands are first carefully examined—namely, the deep inguinal glands, this time from within the abdominal cavity (although by pushing up Poupart's ligament they may also be seen before the abdomen is actually opened). The sublumbar, the renal, the retro-hepatic, and the other glands may in turn be sought for as directed in the lesson on tuberculosis (p. 145).

If nothing is obvious in this direction, the next thing is to cut into the spleen with a knife from the third set of instruments. Holding the spleen with fine forceps, the incision is made across its substance, and with a sterile needle a film preparation is made; or a cover slip may be actually rubbed into the cut surface. The film of splenic pulp is dried, fixed, and stained in the usual way. Anthrax bacilli, and other organisms, are thus very readily and quickly demonstrated, and cultivated, if desired. If, on a similar examination of the other abdominal organs, liver, kidney, &c., no gross lesion is discovered, the thorax is opened, any pleuritic effusion collected in a sterile pipette and examined in the way already mentioned; and lastly, holding the heart by means of the third pair of sterile forceps, the surface is scarified by means of the glass rod, or iron bar, heated to dull redness. A fresh sterile pipette is passed through the scarred area into the ventricle, and the heart blood collected. Cultures may be made on serum and other media from this pipette, and what remains can be examined as a film.

LESSON XVI

GLANDERS. DIPHTHERIA, &c.

A. **Bacillus mallei.**
B. **Bacillus diphtheriæ (Klebs-Löffler bacillus).**

A. I. *Cultivations* on
(i) Glycerin-agar. (ii) Potato.
II. *Stain coverslip preparations*, with—
(i) Löffler's blue. (ii) Anilin-gentian-violet.
(iii) By Gram's method (*the film is decolorised*).

III. *Pus from a diseased animal* may be stained in either of the above ways, II. (i) and (ii), or by the methylene-blue-eosin method employed for gonococci (p. 116).

IV. *Stain sections of lung*, from a case of glanders, by **Noniewicz's method**, as follows:

1. Pour on Löffler's methylene blue. After two to five minutes,
2. Wash in distilled water.
3. Decolorise, according to the thickness of section, from one to five seconds, in a mixture of 0·5 p.c. solution of acetic acid, 75 parts; 0·5 p.c. watery solution of tropæolin, 25 parts.
4. Wash again in distilled water.
5. Spread on slide gently, absorb excess of water with blotting-paper, dry in the air, or in paraffin cupboard at 60° C.
6. Clarify with xylol, and mount in Canada balsam.

The bacilli are deep blue in colour, and the lung itself is of a light blue tint, or may be nearly decolorised. The bacilli are most readily found by looking with the low power for degenerated, or necrotic, and more or less badly staining, areas.

Hewlett[1] recommends staining sections in carbol-methylene blue for half an hour, rinsing in 1 per cent. acetic acid, dehydrating in anilin oil, and clarifying in xylol. The section is mounted in balsam, as usual.

B. I. (*a*) *Cultivations* of each *variety*, *long* and *short* forms, of *B. diphtheriæ*:
(i) Gelatine stab. (ii) Gelatine streak.
(iii) Agar-agar streak. (iv) Glycerin agar-agar streak.
(v) Blood serum. (vi) Broth.

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[1] Hewlett's *Manual of Bacteriology*.

(*b*) *Cultivations* from fresh diphtheritic membrane, on serum.

II. *Stain coverslip preparations* of each variety—

(i) With Löffler's, *or* carbol-methylene blue.
(ii) With carbol-fuchsin.
(iii) By Gram's method.

III. *Stain films*, made by rubbing up a little of the fresh diphtheritic membrane, by the Gram-Eosin method.

IV. *Stain paraffin sections* of diphtheritic membrane, covering the epiglottis, or fauces, by the Gram-Eosin, or Eosin-Gram-Weigert methods.

Glanders

Glanders.—This disease—in France called *morve*, in Germany *Rotz*—which affects chiefly horses, asses, and mules, but by contagion and direct inoculation may also be conveyed to man, is caused by the *Bacillus mallei*. There are two forms of the disease.

One affects the respiratory passages with the formation of minute grey points, which may be readily mistaken for miliary tuberculosis, but really consist of granulomatous tissue, which undergoes necrosis in the centre, the broken-down yellow material closely resembling pus, in which the organisms are readily demonstrated. There is inflammation, with ulceration, of the nasal mucous membrane, accompanied by discharge from the nostrils, which is very characteristic, and is perhaps the most frequent source of contagion for grooms and others in charge of horses, the hand or arm in such cases being generally infected before the nose. This is **glanders** proper.

The other clinical form, known as **farcy**, is characterised by the production of nodules in the skin and subcutaneous tissues, known by the name of **farcy buds**. This form of the disease most frequently appears in the region of the neck and chest, and between the nodules mentioned the lymphatics stand out very prominently. The muscles may be involved, and such foci of disease may give rise to a condition not unlike that of phlegmonous erysipelas, and, in some cases, isolated foci, which, when broken down by degeneration, closely resemble carbuncles (Abbott). Secondary implication of the lungs, kidneys, &c., may follow, the tissues becoming necrosed at the diseased foci, and here the bacilli may sometimes be demonstrated in large numbers. In the discharges, however, the bacilli are found often only with great difficulty. In its acute form, the disease in man runs a fatal course in two or three weeks, with high temperature, delirium, &c. (Hewlett). Every care should therefore be taken when working with cultures or suspected material.

Cultivations.—*Gelatine streak.*—This is not a favourable medium, owing to the low temperature at which it is necessary to keep it. The organism does not develop satisfactorily at a temperature below 24° C., the small amount of growth which does appear being of brown colour. No liquefaction occurs.

Agar-agar.—The growth is moist and fairly well defined. It is of a dull grey colour, and does not rapidly spread over the surface of the medium; in other respects it does not present any characteristic appearance.

Potato.—An exuberant, moist, yellow-wax-like, or honey-like, growth appears in twenty-four hours, darkening in tint to a brown or reddish brown; the growth is very viscid, or mucoid, in consistence.

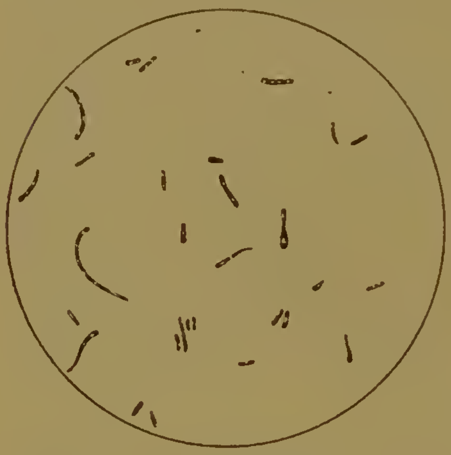

Fig. 83.—GLANDERS BACILLI. × 925
Glycerin agar culture.

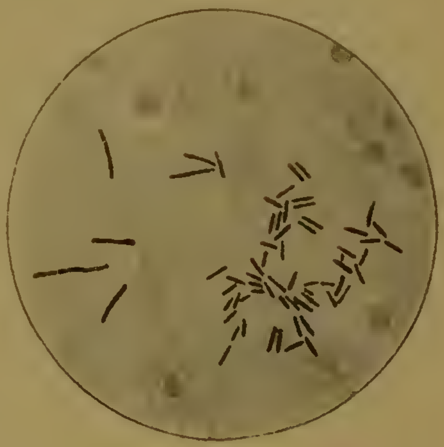

Fig. 84.—GLANDERS BACILLI IN A SECTION OF LUNG. × 925

Broth is speedily rendered turbid, and a viscid deposit forms in a few days. Virulent cultures should be kept always in the incubator at 37° C., as the virulence readily diminishes.

Under the microscope, the organisms are seen, in film preparation (fig. 83), to be rod-shaped bacilli, arranged singly, in pairs, and, occasionally, in threads. The bacillus may be very short, with rounded ends, but one end is frequently pointed, and the other somewhat thickened and clubbed. Instead of being more or less straight, the organism may have its ends curved in opposite directions, like an italic *f*. It stains very irregularly, an appearance which has given rise to some discussion as to spore formation, but there is no direct evidence in favour of there being spores. Amongst the shorter rods, especially in sections of the lung (fig. 84), much longer ones may be seen in places. The organism appears to have a special affinity for Löffler's methylene blue among the simple stains. It is decolorised by Gram's method, thus contrasting with diphtheria bacilli, although no confusion ought to arise if ordinary care is taken in microscopic examination. In sections, however, whatever stain be used, decolorisation of the organism only too readily occurs, rendering its detection in tissues somewhat difficult.

Mallein.—The active poison produced by the glanders bacillus (*B. mallei*) is called *mallein*, and appears to hold the same general relations to that organism as Koch's tuberculin has to the tubercle bacillus—that is to say, when injected into a healthy animal there is little reaction; but if the animal be glandered, in from four to ten or twelve hours, at the point where subcutaneous injection has been made, a severe reaction begins to be manifest, and also great constitutional disturbances, accompanied by fever. Injection with mallein is the means now constantly employed to detect the presence of glanders in suspected horses, the rise of temperature, 2° or 3° C. above normal, &c., serving to indicate the disease unmistakably.

Mallein is prepared by sterilising in a steamer old cultures in 5 per cent. glycerin broth, and then filtering off the bacilli by means of a Pasteur-Chamberland filter.

In the absence of mallein, **Strauss's method** for the rapid diagnosis of glanders may be used. In this method the suspected material, pus, &c., is injected into the peritoneal cavity of a male guinea-pig, which is highly susceptible, the rabbit, however, like the dog, being only slightly susceptible. Rapid swelling of the testicles occurs, and in four or five days they become broken down into caseous masses adherent to the skin, often containing large numbers of the bacilli.

Widal's reaction.—The agglutination of glanders bacilli by the blood of a glandered horse has been described by MacFadyean, as in the case of Widal's sero-diagnosis of typhoid fever.

Diphtheria

Diphtheria.—The clinical features of this disease, due to the **Klebs-Löffler bacillus**, need not be further referred to here.

Cultivations.—*Gelatine stab.*—Minute colonies appear along the track of the needle as discrete white points.

Gelatine streak.—The growth, which takes about three days to develop typically, appears as discrete, and only slightly raised, white colonies, darker and more granular in the centre than at the edges, which are indented and more or less translucent. The growth does not tend to spread much peripherally over the surface of the medium; the gelatine is not liquefied.

Agar-agar.—The growth, in twenty-four hours, consists of separate, rounded, somewhat raised colonies, which, however, are not so prominent as in the case of the serum growth of the same age. In forty-eight hours, the growth,

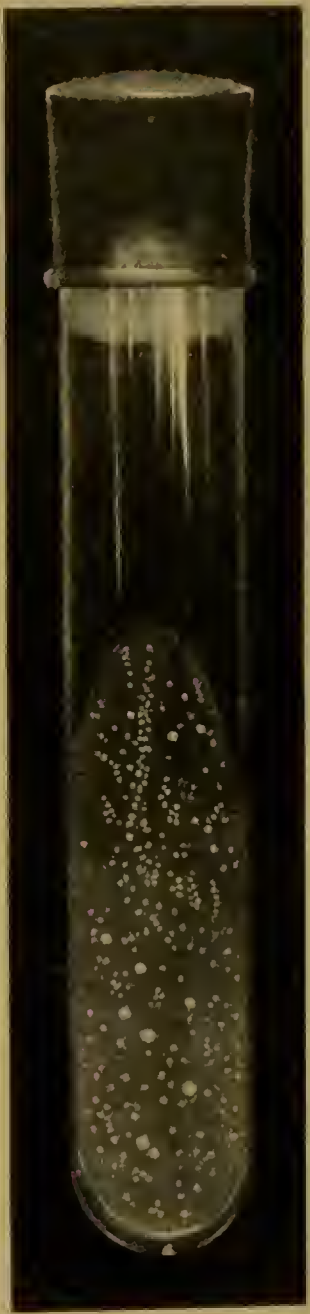

FIG. 85.—BACILLUS DIPHTHERIÆ
Pure culture on serum, about 36 hours old.

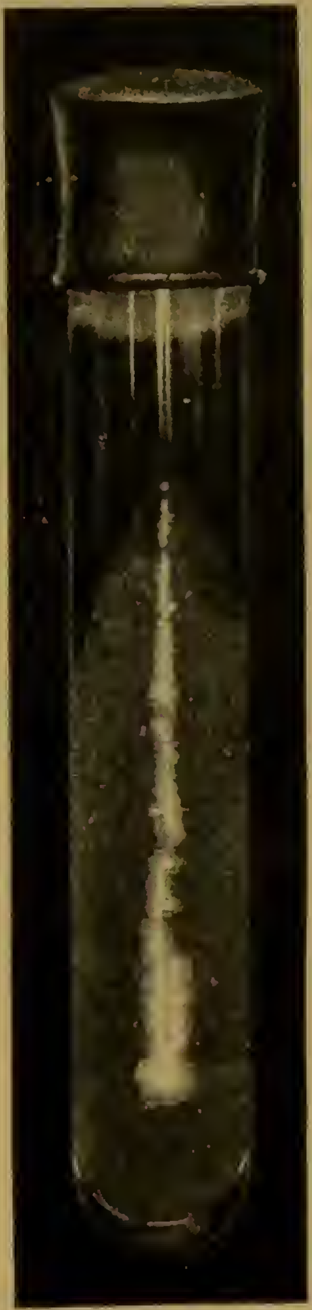

FIG. 86.—BACILLUS DIPHTHERIÆ
Pure subculture on serum, several days old. This raised opaque white streak is less common, unless it is a *sub*culture, and less characteristic than the discrete colonies seen in fig. 85.

owing to the prominence of the discrete colonies above the surface, is fairly characteristic.

Blood serum.—In twelve to sixteen hours, the growth consists of single, somewhat circular, white colonies, thicker in the centre than at the edges, which, examined by the lens, are seen to be a little irregular, and subsequently are distinctly scolloped. These colonies are raised above the surface of the medium at this stage (twelve to sixteen hours), more so than other organisms which would be found in the mouth, as a general rule; so that by picking out these more prominent colonies the diphtheria bacilli can be detected under the microscope, and a pure subculture made at once. In twenty-four hours' time, the growth is still more raised, and opaque white in colour. This is the most characteristic appearance; but it must be added that frequently, *in subcultures*, colonies are not discrete, but run together into an opaque white growth, along the edges of which, however, separate colonies may be seen extending over the surrounding medium; but the general appearance of the growth may sometimes be not unlike that of staphylococcus pyogenes albus massed together, or even that of a young culture of the bacillus coli communis.

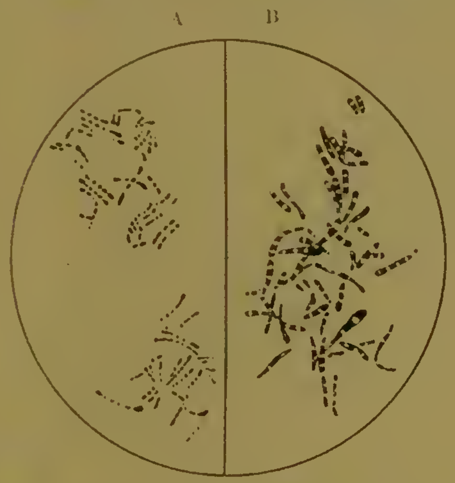

FIG. 87.—BACILLUS DIPHTHERIÆ. TWO SPECIMENS OF THE MEDIUM AND LONG VARIETIES WITH CLUBBED EXTREMITIES

A, ×925. B, ×1,000 (drawn from microphotograph kindly lent by Professor Sidney Martin, F.R.S.) Involution forms are seen in both cases.

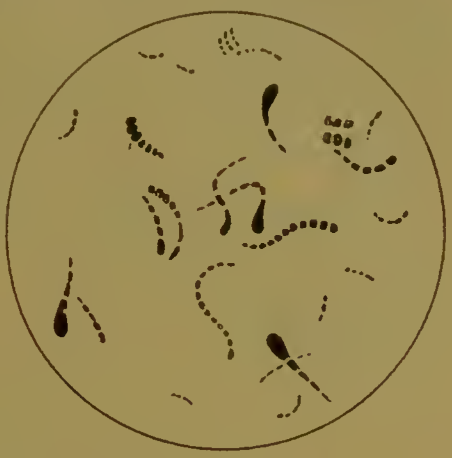

FIG. 88.—BACILLUS DIPHTHERIÆ. ×925

From a culture three months old, showing numerous giant involution forms.

Broth.—Turbidity is produced in less than twenty-four hours; and *the reaction*, if the broth was neutral to start with, *becomes altered* too, so that it *is at first distinctly acid* during the first two days of the growth, and *then returns to its former alkalinity*.

Under the microscope, the Klebs-Löffler bacillus is seen to vary considerably in appearance, so that, occasionally, a little difficulty may be experienced in deciding as to its presence in a culture made from the suspected throat. Most frequently, the bacillus is a long, straight, or slightly curved, non-motile rod, which is thickened, or 'clubbed,' at one or both extremities, and takes the stain irregularly. This irregularity in staining, well demonstrated by the use of Löffler's blue, is due to aggregations of protoplasm bulging the sheath of the bacillus, the clubbed extremities, or poles, being generally more deeply stained than the rest of the bacillus (*polar staining*). The organism is also readily stained by Gram's method. No spore formation occurs.

In most of such cultures larger bacilli are to be seen, in which the clubbing of the extremities is extremely well marked. These are the older 'involution forms,' due to a commencing degeneration; they are very characteristic (figs. 87 and 88), their presence often enabling one to decide with certainty in the case of an otherwise doubtful culture. There are shorter, intermediate-sized bacilli, which have the same general appearance as the long forms already described. The bacilli are frequently curved sinuously, or with the extremities in opposite directions like an italic *ſ*. Sometimes the bacillus is sharply pointed at its extremities, the terminal segments being roughly triangular (fig. 89 A), and arranged with their bases towards each other, connected with each other by the sheath; the bacillus may be made up of two such triangular segments, or between these one or two, more or less oblong, segments may be interposed.

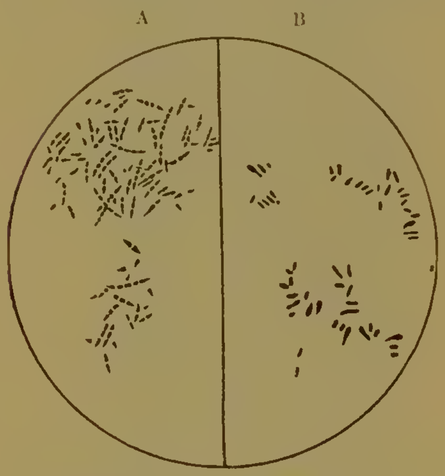

FIG. 89.—BACILLUS DIPHTHERIÆ, × 925

A, long variety, with pointed extremities. B, short form.

Besides more or less elongated specimens of the long varieties just described, there is a definite 'short form' of dipththeria bacillus (fig. 89 B), which appears to be quite distinct from the 'long form,' though some authorities state that the length can be altered by changing the medium used for culture (see p. 161).

The short form of diphtheria bacillus is a short rod with rounded ends, sometimes very slightly curved, and with one end thicker than the other, so that it presents a slightly clubbed appearance. Its outline is well defined, and it stains quite uniformly, the protoplasm not being aggregated into the little masses so characteristic of the long variety. Involution forms, consisting of long, curved organisms, with distinctly clubbed extremities, appear in such cultures, and confirm them to consist of diphtheria bacilli. In old cultures of the long form, the degree of involution that occurs is sometimes very remarkable (see fig. 88).

The relation between the long and the short forms of diphtheria bacilli has not yet been absolutely settled. It was formerly stated (by Abbott, and others), that the appearance of the bacilli varied with the media on which they were grown; so that if blood serum was used for a series of generations the short became converted into the long form; and a bacillus which appeared to be of the long variety when grown on serum, after several generations' growth on glycerin agar-agar, became converted into the shorter and more definitely outlined, and uniformly staining, form.

Lately, however, this view has been questioned by Dr. A. E. Peters,[1] who has kept cultures under observation for a period of two years, and asserts that he has never noted any alteration in the appearance of the bacilli when changed from serum to glycerin agar, and *vice versâ*, the long and short types of bacilli remaining distinct whatever the media used for their cultivation.

This distinction appears to be of importance clinically, for the virulence of the long variety is said to be, broadly speaking, greater, whilst the vitality is somewhat less, than in the case of the short form.

Arrangement of the bacilli.—In examining a film preparation the parallel grouping of the bacilli is very commonly observed, the arrangement being often such as to suggest comparison with the appearance of 'Chinese characters.' Diphtheria bacilli, like other rod-shaped organisms, undergo fission at right angles to their direction, and the diplo-bacillus, or two short rods conjoined, so frequently seen, is thus produced. But parallel grouping of bacilli is so regular a feature in any film that it is evident that division in their length must also occur.

Shattock, discussing this point, says:[2] 'The segmentation of the protoplasm of a rod becomes more and more pronounced until a group of separate elements results, at first not unlike the elements of a streptococcus which have undergone flattening from mutual pressure. In the next place, the separate elements elongate at right angles to what was previously the long axis of the bacillus. One finds all stages between flattened coccus-like forms, and a row of short parallel rods. The rods so resulting lengthen, and they may undergo a similar process of segmentation, and eventually themselves give origin to another series of rods set at right angles to what was once their long axis.'

Examination of diphtheritic membrane.—This may be examined in the fresh condition, a small portion of the membrane being broken off by means of the sterile platinum loop, and rubbed into a coverslip, so as to make a film, which is dried and fixed in the ordinary way. It is, however, better to break up a portion of the membrane in a watch-glass full of normal saline solution, and from the turbidity thus produced to make a film. The bacilli, stained by Gram's method, present the appearances described above; but they may be somewhat thicker, and more irregular, than when taken direct from a cultivation.

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[1] *Trans. Path. Soc.* vol. xlvii.

[2] *Ibid.* vol. xlvi., 1895.

In sections of the membrane, the fibrinous structure is well seen by staining with the Eosin- (or Carmine-) Gram-Weigert's method, which is but a modification of Weigert's special stain for fibrin. Various cocci are seen on the outer surface of the membrane, the unmixed diphtheria bacilli being situated somewhat deeper. When not staining for bacilli, hæmatoxylin can be used to demonstrate the presence of fibrin.

Results of inoculation.—Though solid or liquid cultures may be used, the most convenient way of ascertaining the virulence of the diphtheritic bacillus is to inoculate a medium-sized guinea-pig, of about 250 grams weight, with a forty-eight hours' growth in glucose-broth. A tenth to one or two c.cms. of the fluid generally kills in forty-eight hours; but very small doses of more virulent cultures can kill in from six to eight hours.

At the seat of inoculation of a virulent culture, a characteristic band of induration can be detected after a few hours. At the autopsy, this indurated band is seen to be due to hæmorrhagic œdema, in which the specific bacillus can be readily demonstrated. The neighbouring lymphatic glands are swollen and congested. There is an excess of œdematous fluid, which may be blood-stained, in the pleural and peritoneal cavities, and the congestion of the omentum, &c., is sometimes so acute that it may be described as hæmorrhagic peritonitis. The suprarenals, in acute cases, are of deep crimson tint, the spleen may be a little congested and swollen, and there are petechiæ on the surface of the liver.

Only in rare cases is the bacillus found in the tissues generally, beyond the set of lymphatics nearest the point of inoculation. Pure cultures from the heart's blood can, however, sometimes be obtained. Experimentally, with cultures of highly exalted virulence, the bacillus has been found,[1] by Bulloch and others, even in the liver. But, as a rule, the blood and viscera are not invaded by the organism, which is confined to the seat of inoculation, where it brews a soluble **toxalbumin**, allied in nature to snake venom.

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[1] Captain Frederick Smith, R.A.M.C., D.P.H., has found virulent diphtheria bacilli in the urine five hours after experimental inoculation of a guinea-pig. with, or without, hæmaturia, an important point for hygienists, possibly explaining the connection between general insanitation, middens, and epidemics of diphtheria. As in the similar case of typhoid bacilli in urine (p. 172), all the excreta, solid and liquid, should therefore be disinfected. (*Lancet*, November 19, 1898.)

In its distribution, and in its mode of action, therefore, the bacillus diphtheriæ is comparable with that of tetanus.

Mode of infection.—Diphtheria is at first a purely local infection, the bacilli, as already mentioned, being as a rule confined to the seat of inoculation, not spreading into the tissues beyond the nearest lymphatic glands, or entering the circulation. Even when injected into the blood, the bacilli themselves soon disappear. The disease, however, continues to spread, as the result of the formation and absorption into the system of the chemical products, or toxins, brewed by the specific organism; it is essentially a *toxæmia*.

This may be readily demonstrated by injecting a guinea-pig with a small quantity of the *germ-free* fluid obtained by passing through a Berkefeld or Chamberland filter a forty-eight hours' old broth culture of the Klebs-Löffler bacilli obtained from a case of diphtheria. Such filtered fluid is absolutely sterile, and yet its inoculation produces the same pathogenic effects as would follow the use of a pure culture of the bacillus, including, for instance, diphtheritic paralysis; whilst a larger dose produces a fatal result.

The consideration of the question of immunity and immunisation may be conveniently left to the end of the present chapter.

The 'pseudo-diphtheritic' bacillus of Löffler, and the 'pseudo-diphtheria' bacillus of von Hoffmann.—Löffler in 1887, and Hoffmann in 1888, drew attention to the presence of non-virulent organisms possessing microscopic and cultural characters closely resembling those of true diphtheria (Klebs-Löffler) bacilli. Yet, with care, they were able to distinguish the one from the other, even though occasionally, but not invariably, they were found associated with one another.

Such statements naturally aroused a great controversy, which has not yet been satisfactorily settled, in spite of an ever increasing literature. One of the most important recent contributions to this highly practical question is by Dr. Hewlett and Miss Knight, M.B.,[1] which may also be consulted for its excellent summary of previous observations.

The 'pseudo' organisms referred to have been found associated with Klebs-Löffler bacilli in cultures taken from cases of undoubted diphtheria at all stages; alone, or mixed with the true organism, in perfectly healthy-looking throats of attendants on cases of diphtheria; and in various forms of non-diphtheritic throat anginas, in measles, scarlet fever, pharyngeal catarrh; and especially in membranous rhinitis. These cases, clinically, are distinguishable by their non-spreading and non-contagious characters, membranous rhinitis, for instance, being in these ways readily distinguished from nasal diphtheria.

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[1] *Trans. Brit. Inst. Preventive Medicine*, first series, 1897.

Microscopically, it is admitted, by all, that the so-called pseudo-diphtheritic bacillus closely resembles the short form of the true diphtheria organism, but those[1] who advocate the existence of a definite 'pseudo' bacillus state that it is thicker at the centre than at the ends, 'plumper,' shorter, and less variable than the Klebs-Löffler bacillus, and that involution forms are rare. Further, they stain more deeply and regularly than the Klebs-Löffler bacillus, and polar-staining, common in the latter, is rare.

Cultivations.—*On potato,* rendered alkaline before sterilisation by 10 per cent. solution of sodium carbonate, the growth in the case of the Klebs-Löffler bacillus is almost invisible, whereas in the case of the pseudo organism there is a distinct creamy growth in two days.

When grown on neutral litmus agar,[2] the purple tint changes to a blue, indicating the formation of alkaline products, in the case of the pseudo-diphtheritic bacillus; whereas in the case of true diphtheria, a strongly acid reaction is evidenced by the bright red tint of the medium, even in twenty-four hours. These and other distinctions mentioned in the paper by Dr. Hewlett and Miss Knight are conveniently tabulated by them as follows:

	Pseudo-diphtheria bacillus	Klebs-Löffler bacillus
Morphology	Rods 1μ to $1{\cdot}6\mu$ in length, tending to be slightly thicker at the centre than at the ends. Is 'plumper,' shorter, and less variable than the Klebs-Löffler bacillus. Involution forms rare	Rods averaging $1{\cdot}2\mu$ to 2μ in length. Slender and of the same diameter throughout. Considerable variation in size. Involution forms usually present
Staining	Stains more deeply and regularly than the Klebs-Löffler bacillus. Polar-staining rare	Staining generally more or less irregular, and polar-staining common
Alkaline potato	Distinct, cream-coloured colonies, or growth, visible in two days	Grows well, but growth is almost invisible
Neutral litmus agar	Alkaline reaction	Acid reaction
Stab cultures in sugar-agar, and gelatine	Growth only at upper part of stab	Growth along whole length of stab
Anaërobic cultures in hydrogen (nutrient broth)	No growth	Grows well
Indol reaction (*peptone-water*[3] cultures, with sulphuric acid alone)	Only after three weeks' growth	After one week's growth

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[1] *Trans. Brit. Inst. Preventive Medicine,* first series, 1897.

[2] See p. 10.

[3] It is essential to use peptone-water, and not broth cultures, to obtain this distinction, which ceases to exist also if a nitrite be added. The original paper should be consulted.

Perhaps *one of the best media* for distinguishing the two bacilli is *alkaline nutrient broth*, kept at 37° C. In the case of the pseudo bacillus, the broth does not become so soon cloudy, the precipitate formed being usually more finely granular; the change in reaction from the original alkalinity to the acid condition is slighter than in the case of the Klebs-Löffler bacillus (see p. 160), and then there is a return to that alkalinity subsequently.

These differences, so sharply brought out in the above table, would at first sight seem to establish the separate existence of the 'pseudo' as distinguished from the true diphtheria bacillus. Those opposed to this view—notably Roux and Yersin, Abbott, and others—believe, however, that most of the 'pseudo' forms are merely diphtheria organisms which have, by some means or other, lost their virulence, it being well known that undoubted diphtheria bacilli vary within extremely wide limits in their virulence and pathogenic properties generally; and in this respect the varying pathogenicity of the streptococcus and other coccus forms may well be borne in mind.

It must be admitted that evidence favouring Roux's and Abbott's views would seem to be derived from other observations of Dr. Hewlett and Miss Knight to the effect:—

(i) that the 'pseudo' bacillus seems to replace the diphtheria bacillus in some cases of diphtheria as they progress towards convalescence.

(ii) that though 'pseudo' forms occur but very rarely in cultures of the Klebs-Löffler bacillus, 'Klebs-Löffler or diphtheritic forms' occur almost always, some time or other, in cultures of the 'pseudo' bacillus, and in a number of cultures it is possible to obtain a series of organisms forming a connecting chain between the Klebs-Löffler and the 'pseudo' bacillus.

(iii) that it has apparently been possible, by careful heating, to convert a typical virulent Klebs-Löffler into a typical, non-virulent, 'pseudo' bacillus; whilst, by cultivation and incubation, and passage through an animal, a 'pseudo' has apparently been converted into a Klebs-Löffler bacillus.

The authors quoted therefore finally conclude 'that the "pseudo" is sometimes a modified Klebs-Löffler, though perhaps not always, as possibly more than one species having the same morphology may exist.'

The really important point is to note that organisms practically identical in appearance with virulent diphtheria bacilli may be present in various clinical conditions, and their pathogenicity can only be verified by the effects of inoculation (see p. 162).

The xerosis bacillus of Neisser is found associated not only with xerosis of the conjunctiva, but also in some cases of follicular conjunctivitis, trachoma, chalazion, and even in quite a large proportion of healthy conjunctival sacs, according to some observers.[1]

Its chief interest is on account of its close morphological, cultural, and staining relationships with the Klebs-Löffler bacillus. Fränkel, indeed, included it among the pseudo-diphtheritic bacilli, which it resembles in being non-virulent when injected into guinea-pigs; few or no clubbed forms are seen, and when grown in neutral or alkaline broth no acid reaction occurs.

Though *sub-cultures* grow, roughly speaking, as rapidly as in the case of the Klebs-Löffler bacillus, no growth of the xerosis bacillus appears for thirty-six to forty-eight hours when the original culture is made from the eye.

For further details, see a recent paper by Dr. Eyre.[2]

A smaller, but otherwise similar, bacillus, morphologically, has been recently shown to be present in **whooping-cough**.

Immunity and immunisation

As has been stated already, diphtheria can be experimentally produced by inoculating either the specific bacillus, or the toxin brewed by it and passed through a filter, so as to be germ-free.

If the amount of injected solid or fluid culture, or of germ-free toxin, be less than the fatal dose (generally spoken of as a *sub-lethal* dose), the animal on recovering its health is found to have acquired a certain amount of immunity against subsequent inoculations, the degree of immunity depending on the virulence and amount of the material employed.

By the use of a series of such inoculations, steadily increasing the amount or virulence of the cultures, or toxins, or both quantity and virulence of culture and toxin mixed, practically complete immunity can be conferred on an animal. The blood serum of such an animal has the remarkable property of conferring immunity when injected into other animals, so that, in appropriate doses, it protects them from subsequent infection by the specific organism or its toxin; and if injected within a certain time after inoculation with that organism it can successfully counteract its otherwise fatal effects.

In other words, the serum from an animal immunised against the diphtheria bacillus or its toxin, or both, is preventive and curative when injected into other animals; and what has been said of the Klebs-Löffler bacillus is equally true of the organism of tetanus. Such immunising serums are generally spoken of as '**antitoxins.**'

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[1] Lawson, *Brit. Med. Journal*, vol. ii., 1898, p. 186.

[2] Eyre, *Trans. Path. Soc. Lond.*, 1896.

When recovery occurs from disease naturally, or artificially, acquired, it is the result of an active struggle on the part of the animal's defensive agencies, whatever they may be, with the organisms or toxins introduced. The **immunity** so conferred is said to be '**active**;' whilst that obtained by subsequently inoculating other animals with the serum of this actively immunised animal is said to be '**passive**.' Active immunity is slowly acquired, after more or less severe general reaction, but is then more permanent than the passive form, resulting immediately, and with little or no constitutional disturbance, after the use of the immunising serum.

Amongst the defensive agencies at work in the body may be mentioned normal blood serum, which is actively germicidal, and it is also thought that the leucocytes may secrete fluids which to a certain extent neutralise poisonous toxins, and then the organisms themselves are more readily dealt with by the phagocytes. Besides the blood serum and the leucocytes, it seems highly probable that the tissue cells, generally, are stimulated to unusual activity in the presence of toxic products, resulting in the formation of certain '**protective proteids**,' and these appear to be the most important of all the agencies at work.

Diphtheria antitoxin

Diphtheria antitoxin.—This is prepared at the farm of the Jenner Institute as follows.[1] A horse, proved free from glanders and tubercle, is injected, generally beneath the skin over the scapula, with the toxins derived by filtering an eight-days' broth culture of highly virulent diphtheria bacilli. The dose is repeated from time to time, being gradually increased, starting with a few cub. cms. and going up to 200 or 250 c.c. At the seat of injection a swelling nearly the size of an orange may be observed, and there is often a sharp rise in the temperature and other manifestations of reaction, which subside after two or three days until the next injection is given.

When the animal ceases to react to further injections, blood from the jugular vein is drawn off under strictest aseptic precautions, the blood and serum being then allowed to separate. The protective properties of the serum are then ascertained by mixing it in different proportions with ten times the minimal lethal dose of a standardised sample of diphtheria toxin, and injecting these mixtures into a series of guinea-pigs, each about 250 grammes in weight.

Anti-toxin units.—The amount of serum found necessary to neutralise ten times the minimal lethal dose of toxin is termed a '**unit**,' but it is more convenient for practical purposes to have a unit ten times as large as the one just mentioned, and this is called a 'normal unit.'

A normal unit ('N.U.'), then, is ten times the amount of serum required to completely counteract the effect of ten times the minimal lethal dose of diphtheria toxin in a guinea-pig weighing about 250 grammes.

Thus suppose the minimal lethal dose of standard toxin be $\frac{1}{10}$ c.c., ten times the minimal lethal dose will be 1 c.c. If the smallest quantity of serum which will neutralise 1 c.c. of toxin is found to be $\frac{1}{1000}$ c.c., then ten times this amount of serum, which is the so-called normal unit = $\frac{1}{100}$ c.c. In other words, 1 c.c. of the serum contains 100 N.U., and so on.

---

[1] More rapid methods of preparing antitoxin, and of obtaining it of very high potency, have been discovered; but for further details larger treatises and recent medical journals should be referred to; the exhaustive article by the late Professor Kanthack in vol. ii. of Clifford Allbutt's *System of Medicine* will well repay careful perusal.

The normal unit is a purely arbitrary standard, and many suggestions for its improvement have been made.

The dose of diphtheria antitoxin ranges from 1,500 to 3,000 or even 6,000 N.U., according to the severity of the case. Some give the smallest dose mentioned, repeating every twelve hours; whilst others prefer to give one or two large doses, repeating only if specially indicated. Most observers are agreed both as to the great value of this method of treating diphtheria, and to the necessity for its use at the earliest moment.

The report of the committee appointed by the Clinical Society of London, 'On the Antitoxin of Diphtheria,'[1] proves conclusively that by the use of antitoxin—

1. The general mortality is reduced by one-third.
2. The mortality in tracheotomy falls by one-half.
3. Extension of the membrane to the larynx very rarely occurs after the administration of antitoxin, which even when used in very large doses produces no serious ill effects; rashes occur in about one-third of the cases; but, like the pain and swelling about the joints occurring in a small number of cases, are of quite temporary duration.
4. The frequency of the occurrence of paralysis resulting from the toxæmia of diphtheria is not diminished, but the percentage of recoveries in cases with paralysis is slightly increased.

LESSON XVII

A. Bacillus typhosus
B. Bacillus coli communis

I. *Cultivations* of A and B:
  (i) Broth.
  (ii) Peptone-water.
  (iii) Milk. Examine daily, and note which coagulates the milk.
  (iv) Gelatine (α) Stab.
    (β) Streak.
    (γ) 'Shake.' Examine daily, and see which forms gas.
  (v) Agar-agar (α) Streak. Note daily which appears to be the more vigorous growth.
    (β) Plates (three). Examine daily with ⅔-inch objective.

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[1] *Trans. Clin. Soc. Lond.*, 1898.

(vi) Litmus sugar-agar: 'Shake.' Note the change in colour, and observe which culture forms gas.

(vii) Potato.

(viii) Potato-gelatine (α) Streak.

(β) Plates (three), made, in the usual way, from half a test-tubeful of sterile water in which one small loopful each of A and B has been thoroughly mixed. Examine daily (α) and (β)—the plates, by means of the $\frac{2}{3}$-inch objective.

II. *Stain coverslip preparations* of A and B:

(i) Carbol-fuchsin;

(ii) Anilin-gentian-violet;

(iii) Gram's method (*both organisms are decolorised*).

III. *Make a hanging-drop* from a fresh broth culture, preferably not more than twenty-four hours old. Note the difference in the motility of the two organisms, A and B.

IV. *Do the 'indol reaction'* with the peptone-water culture of A and B, after twenty-four to forty-eight hours' incubation (the longer period is generally advisable), as follows:

To 5 c.c. of the peptone-water (or broth) cultures of A and B add 1 c.c. of a 0·01 per cent. solution of sodium or potassium nitrite. Pour in a few drops of concentrated sulphuric acid, when, if any indol is present, a pink colour appears at once, or on standing for a short time.

Note which of the two (A, or B,) cultures gives this indol reaction. It is better to do this test with peptone-water cultures, though broth may be used.

V. *Flagella-staining.*—Cultures of A and B on glycerine agar, ten to eighteen hours old, may be stained by the following methods.

Preliminary preparation (for all methods)

(*a*) Commencing with the culture of typhoid bacilli, a loopful is to be diluted with a watch-glassful of tap-water or normal saline solution, either of these being less harmful to the delicate flagella than distilled water.

The safer and better way is to mix the loopful of culture with half a test-tubeful of tap-water, rotating the tube in a vertical position steadily, as in the case of a 'shake-culture' (p. 21), so as not to injure the flagella, which readily drop off from the bacilli to which they belong.

(*b*) The coverslips are to be carefully freed from fat, &c., by boiling in the bichromate of potash solution recommended by Van Ermengem.[1]

(*c*) The film is to be prepared by placing a loopful of the culture, diluted as directed above, in the centre of the clean coverslip, held in cornet forceps. The coverslip is tilted so as to spread out the drop in various directions. On no account is the platinum loop to be used to distribute the drop, in the way ordinary films are spread, as this would cause the detachment of many of the flagella from the bacilli to which they belong.

(*d*) The film is dried in the air, or in the warm incubator, or high up over a Bunsen flame. It is fixed by passing rapidly through the flame, but the coverslip should be held in the fingers, to prevent the risk of overheating during this process.

With the film prepared as above, the following methods may be tried:

1. **Van Ermengem's method.**

Solutions required

A mordant, 'α.'

Osmic acid, 2 per cent. solution	1 part.
Tannin, 10 to 25 per cent. (*use* 20 per cent.)	2 parts.

To each 100 c.c. of this mixture add four or five drops glacial acetic acid.

A 'sensitising bath,' 'β.'

Nitrate of silver (·25 to ·5 per cent. solution).

A 'reducing and reinforcing bath,' 'γ.'

Gallic acid	5 grammes.
Tannin	3 „
Fused acetate of soda	10 „
Distilled water	350 c.c.

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[1] The formula of this solution is—

Potassium bichromate	60 grammes.
Concentrated sulphuric acid	60 c.c.
Water	1,000 c.c.

Then wash repeatedly in water.

Keep the coverslips in absolute alcohol.

Before use, remove with forceps, and allow them to dry, without wiping, by placing them in a vertical position, protected from the dust.

N.B.—Boiling in nitric acid (p. 13) yields equally good results in most cases.

Van Ermengem's method

(i) Pour a few drops of 'α' solution on to film, and allow to act for half-hour (at 60° C., five minutes will be sufficient).

(ii) Wash very carefully in large excess of distilled water and then in absolute alcohol.

(iii) Now keep it for three to five seconds in β solution.

(iv) Without washing, pass quickly through γ solution.

(v) Wash again in fresh quantity of β solution, moving specimen about gently, and withdrawing it when the solution begins to turn black.

(vi) Wash it thoroughly in several changes of distilled water.

(vii) Dry it carefully between blotting-paper.

Mount first in water, and examine with water-immersion lens (see below).

If the specimen be satisfactory, mount in balsam.

If the flagella be not sufficiently stained, float off the coverslip and start again at (iv).

Care must be taken to change the silver nitrate as soon as any precipitation shows itself.

For some unknown reason, possibly differences in the chemicals used by different workers, slight modifications of the original method have been found either necessary or advantageous.

Van Ermengem's method, modified as follows, may be recommended as giving, with a little practice, uniformly good results:

(i) The film is covered with solution α, either for one hour in the cold, or for fifteen to twenty minutes in the paraffin cupboard at 60° C.

(ii) The coverslip is gently, but thoroughly, washed in distilled water, until all the black colour has gone; and it is then drained dry on blotting-paper.

(iii) Place in absolute alcohol for five minutes. Drain dry on blotting-paper.

(iv) Cover the film with solution β for two minutes. Drain dry, without washing.

(v) Pour on solution γ; after five minutes, drain dry.

(vi) Place in watch-glassful of solution β; move about constantly until the solution begins to get black.

(vii) Wash well in several changes of distilled water, drain and dry gently with blotting-paper, or in the paraffin chamber, and mount in water. Examine with the $\frac{1}{12}$-inch oil-immersion lens, using water instead of oil. If the flagella are seen well stained, dry the coverslip and mount in Canada balsam. If insufficiently stained, transfer the coverslip to solution γ, and repeat stages (v) and (vi).

2. Pitfield's method

Solutions required

A. Tannic acid	1 gramme
Distilled water (cold)	10 c.c.

Filter and keep in bottle.

B. Solution of alum, saturated in cold water	10 c.c.
Gentian-violet, saturated alcoholic solution	1 c.c.

Filter and keep in bottle.

Pitfield's method is as follows:

(i) Immediately before use, mix equal parts of A and B, and cover the film with the mixture.

(ii) Hold the film in cornet forceps and warm over a small flame, till the fluid nearly boils. Lay the film aside.

(iii) At the end of a minute, wash in water.

(iv) Filter on some anilin-gentian-violet solution, but pour off after a second, as recommended by Hewlett.

(v) Wash, dry, and mount.

This simple and very rapid method gives fair results, though the flagella are not so sharply photographed as in the case of Van Ermengem's method.

Bacillus typhosus and Bacillus coli communis

Bacillus coli communis, originally known as the *Bacterium coli commune*, or as *Escherich's bacillus*, after the name of its discoverer, is one of the commonest organisms in the normal intestine, being present everywhere from stomach to rectum, appearing shortly after birth, and before food has been given the child (Escherich). It so closely resembles the bacillus causing typhoid fever, **B. typhosus**, or *Eberth's bacillus*, that the real identity of the two is still more or less under discussion, some stating that *B. typhosus* is simply a more virulent form of *B. coli*.

It may here be mentioned that where *meteorism* is a marked feature in the severer forms of typhoid fever, there is a very notable associated increase in the number of *B. coli* present: and this organism is said to be the commonest cause of *cystitis*.

In a certain proportion of cases of typhoid fever, at varying periods, typhoid bacilli are excreted with the urine, which therefore requires to be as carefully disinfected as in the case of typhoid stools.

The various cultures and chemical reactions which have been made in the above lesson will serve to indicate some of the chief points of difference between the two, and these may be tabulated as on p. 174, *et. seq.*

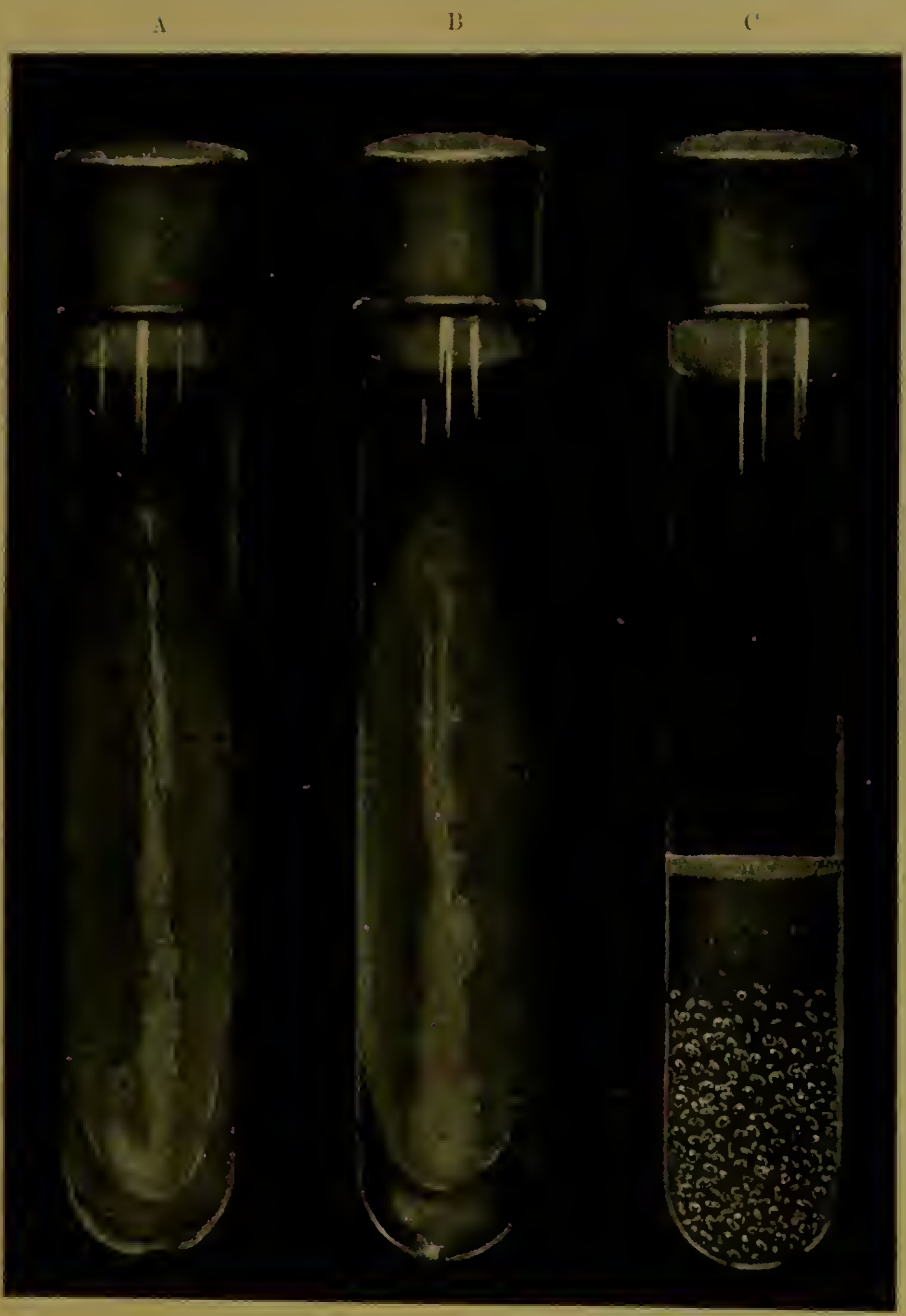


FIG. 90.—A, BACILLUS TYPHOSUS; B, BACILLUS COLI COMMUNIS; C, BACILLUS COLI COMMUNIS

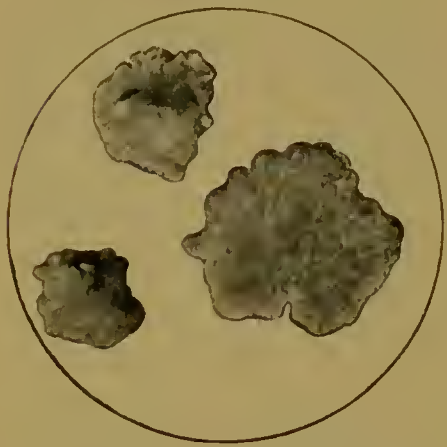
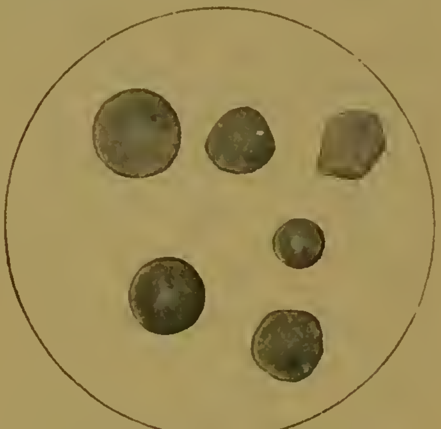
A and B, glycerine-agar streak cultures, 48 hours old, at 37° C., for comparison. (N.B.—The growth in the case of *B. coli* is generally even more diffuse than is shown.) C, gelatine 'shake' cultivation, 24 hours old, kept at 22° C. Note the dotted colonies above the gas bubbles, and the surface growth.

CULTIVATIONS

B. typhosus	**B. coli communis**
Broth.—In twenty-four hours, the turbidity is marked. The reaction, in thirty-six to forty-eight hours, is hardly altered, compared with the broth before inoculation.	*Broth.*—In twenty-four hours, the turbidity is slightly more marked than in the case of *B. typhosus*. The reaction becomes distinctly acid.
Peptone-water.—Some turbidity is present after twenty-four hours. It is not so marked as in the case of *B. coli*, and it does not give the indol reaction, however long it is kept.	*Peptone-water.*—The turbidity is marked; and, after thirty-six to forty-eight hours, it gives a well-marked indol reaction. This becomes the more marked the longer the peptone-water has been incubated at 37° C.
Milk.—Coagulation does not occur.	*Milk* is coagulated throughout, in from thirty-six to forty-eight; or, in some cases, after seventy-two hours. Sometimes, though very rarely, the coagulation is less marked; but the formation of acid without coagulation has been observed.
Gelatine stab.—There is a scanty white growth along the track of the needle, and on the surface the medium becomes a little opalescent. No liquefaction of the gelatine.	*Gelatine stab.*—Along the track of the needle an irregular chain of small, somewhat translucent spherical colonies, of white or bluish-white tint, appears in twenty-four hours. Later, they become of yellowish-white colour. There is a thick, dirty greyish-white surface growth. No liquefaction of gelatine.
Streak.—The growth is white and moist, but not very exuberant. The margins are well defined, but the growth does not tend to spread laterally, as in the case of *B. coli*.	*Streak.*—The growth is more exuberant, and tends to spread laterally more rapidly than in the case of *B. typhosus*. It is moist, and has at first a pale bluish-white, almost fluorescent appearance, then becomes of a dirty greyish-white tint.
‘*Shake.*’—The gelatine becomes a little turbid from the development of minute colonies in the depth. On the surface there is a thin, slightly opalescent growth. No formation of gas occurs.	‘*Shake.*’—The gelatine becomes a little turbid, from the development of minute colonies in the depth, and similar colonies, forming a dirty greyish growth, soon spread to the periphery. Numerous gas bubbles form (fig. 90, c) after incubation at from 20° to 22° C., overnight. This gas is a mixture containing hydrogen and carbon dioxide.
Agar-agar streak.—The growth is greyish-white and moist; the edges are somewhat irregular, or small discrete circular colonies may be dotted along the margins. There is less tendency to spread over the surface of the medium than in the case of *B. coli*.	*Agar-agar streak.*—The growth is at first similar to that of *B. typhosus*. The margins are somewhat more crenate than in the case of the typhoid bacillus. Owing to the greater exuberance of the growth it rapidly spreads laterally over the surface of the medium, differing from *B. typhosus* in this respect.

B. typhosus	**B. coli communis**
Agar-agar plates.—The colonies, which appear in eighteen to twenty-four hours, as seen by the naked eye, are opalescent, somewhat circular dots. When examined under the microscope, with a ⅔-inch objective, the colonies are seen to be irregular, often roughly triangular, or polygonal, in their outline. In the central, denser, portion there often appears a circular spot looking like a nucleus, but apparently a fragment of the original growth with which the plate was inoculated, and which has given rise to the colony under observation. The peripheral portion of the colony is more translucent and of a faint yellow colour. The surface of the colony generally is granular, or finely striated. On focussing the striæ, ridges and cracks may be seen, especially when viewed by refracted light. The appearance presented has been well compared to that of a relief map, showing mountains and dales (see fig. 91).	*Agar-agar plates.*—The colonies, which appear in from eighteen to twenty-four hours, are opalescent, circular dots, of a light buff colour, when seen under the microscope. The colonies are often more sharply defined and circular than in the case of *B. typhosus*. They are also of a browner tint and more coarsely granular, the central portion being of a darker hue, and somewhat marked off from the periphery (fig. 92). The surface colonies, as is so frequently the case, are larger than those in the depth, where they are seen in profile, or optical section, to possess the shape of a biconvex lens. Similar lens-shaped colonies, but lighter in tint, are seen in the case of *B. typhosus*; but they are so frequently observed that they cannot be regarded as in any way characteristic of either organism.
Neutral litmus glucose agar-agar 'shake.'—The litmus does not turn red. No gas formation.	*Neutral litmus agar 'shake.'*—Tube filled with large gas-bubbles after twenty-four hours' growth at 37° C. Litmus turned bright red in twenty-four to forty-eight hours.
Potato.—The growth in twenty-four hours is hardly visible, except on the most careful examination, rotating the tube so as to view the surface of the medium obliquely. It is not unlike the appearance of a snail's track. In some cases of, undoubtedly pure, typhoid cultures the growth is much more obvious, indeed, not altogether unlike some of the lighter cultures of *B. coli*.	*Potato.*—The growth in twenty-four hours is considerable and of a dirty fawn colour. It is raised and moist, and soon spreads over the surface. Sometimes, instead of getting darker, the colour is from the first lighter in tint than indicated above, and remains so.
Potato-gelatine streak (kept at 22° C.).—In forty-eight hours the growth is hardly perceptible to the naked eye, unless looked at by oblique illumination, when the needle track just becomes visible, owing to the very minute colonies which form a fine haze, or nebulous growth. In some cases the growth is a little more obvious.	*Potato-gelatine streak.*—In twenty-four hours considerable growth is seen, in marked contrast to that in the case of *B. typhosus*. The colonies are very minute, circular, translucent dots.
Potato-gelatine plates.—For about the first forty-eight hours the colonies	*Potato-gelatine plates.*—In twenty-four hours colonies are readily seen; they

CULTIVATIONS (*continued*)

B. typhosus	**B. coli communis**
are hardly perceptible by the unaided eye. Under a low power of the microscope, the minute, clear, rounded colonies may be seen, the surface being either structureless, or very finely granular. After sixty hours' growth, the colonies become visible to the naked eye. Under a low power of the microscope they	are circular, or ovoid, more coarsely granular, and somewhat darker, than the corresponding ones of *B. typhosus* would be after forty-eight hours' growth. The colonies in both cases are always more developed on the surface than in the depth, where they are frequently seen in optical section as biconvex lens-shaped bodies (see p. 175).
 FIG. 91.—BACILLUS TYPHOSUS Potato gelatine culture (viewed by refracted light) after sixty hours' growth. The 'relief map' appearance, referred to in text, is well seen.	 FIG. 92.—BACILLUS COLI Potato gelatine culture (viewed by refracted light) after sixty hours' growth.
are then seen to possess a faint yellow tint; they are semi-translucent, the edges are irregular, the surface is finely striated. When examined carefully by refracted light, the surface is frequently seen to be raised into mounds separated by depressions (fig. 91)—the striæ just referred to producing the appearance of a 'relief-map,' as in the case of agar-agar plates. If the surface is more granular it is not always possible to distinguish it from a colony of *B. coli*.	After sixty hours' growth, the colonies become larger, darker, and more numerous. The general appearance of the surface remains much the same as before.

It should be clearly understood that cultures of these two organisms are frequently met with which do not give all the characteristic reactions just tabulated. In these cases one must be content if the majority of the tests can be demonstrated. Thus there appear to be *varieties of B. typhosus and B. coli*, with a group of organisms forming a link between these two series, reacting, partly like one and partly like the other. Refer, also, to p. 189.

In practice, **the most important culture tests** for *B. typhosus*, distinguishing it from *B. coli*, are, unfortunately, of a negative character. They are, in the case of *B. typhosus*—

(i) The non-formation of gas;
(ii) The non-coagulation of milk;
(iii) The absence of the indol reaction; and
(iv) The almost imperceptible, glazelike, growth on potato.

For laboratory purposes it will be convenient to discuss the microscopic appearances of film preparations of these two organisms together.

Bacillus typhosus.—Under the microscope, the organism appears as a short, fat rod, with rounded ends (fig. 93), the length varying, but being generally three times that of the breadth. According to Delépine, the diameter averages from $0\cdot7\mu$ to $0\cdot9\mu$, compared with $0\cdot3\mu$ to $0\cdot6\mu$ in the case of *B. coli*. Very long threads are seen, especially in broth cultivations. These may show no signs of division, but are frequently segmented up into shorter rods, sometimes forming a long chain. Eventually these chains break up into individual bacilli. The protoplasm is, generally speaking, uniformly stained, but in most preparations there is an appearance of vacuoles in many of the bacilli which does not indicate the presence of spores, but is most probably due to the shrinking of the protoplasm from the sheath, and its aggregation into little masses—a process known as *plasmolysis*.

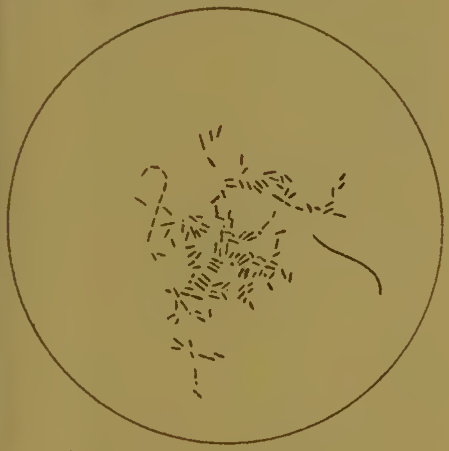


FIG. 93.—BACILLUS TYPHOSUS × 530
Glycerine-agar culture, twenty-four hours old.

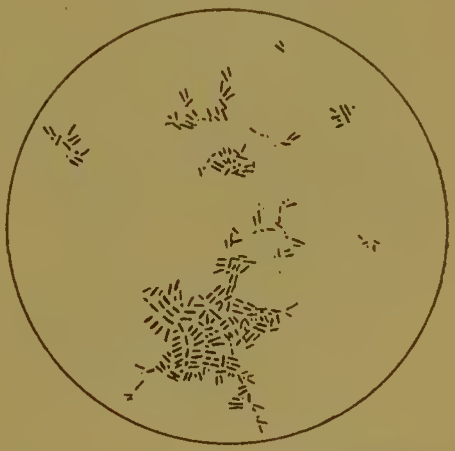


FIG. 94.—BACILLUS COLI COMMUNIS × 530
Glycerine-agar culture, twenty-four hours old.

The bacillus is decolorised by Gram's method of staining, though readily taking the simple stains carbol-fuchsin, Löffler's blue, &c.

Flagella. The organism is seen to possess whip-like *flagella* (fig. 95, A), varying in number from twelve to sixteen. These are frequently broken off during the staining process, from six to eight, or ten, being the number usually seen. The flagella are very fine, and from four to five times the length of the bacillus to which they are attached.

The hanging-drop shows the typhoid bacilli moving with great rapidity across the field of the microscope, the movement being eel-like in character. This darting, wriggling motion is a great contrast to what is seen in the case of *B. coli* as a general rule, and probably partly depends on the greater number of flagella which it possesses. Typhoid bacilli maintain their activity almost undiminished, in a properly prepared hanging-drop, for over thirty hours.

Bacillus coli communis.—Under the microscope, this organism exhibits great variety in length, being sometimes hardly longer than it is broad, so as to appear like a rather large coccus; but, as a general rule, it is a short, fat bacillus, indistinguishable from *B. typhosus*. Long threads are less frequently seen than in the case of typhoid bacilli. It is decolorised by Gram's method of staining.

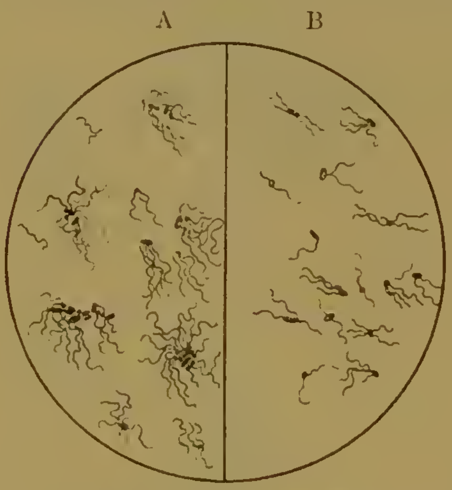


FIG. 95.—FLAGELLA STAINING, VAN ERMENGEM'S METHOD. × 530

A, bacillus typhosus; B, bacillus coli communis. Twenty-hour-old cultures on glycerine agar.

Flagella.—When specially prepared by one of the methods already mentioned, the bacillus is seen to have from six to eight flagella; but more frequently the number to be actually counted is from two to four. The flagella appear to be detached more readily in the case of *B. coli* than in the case of *B. typhosus*; and, whilst it is possible to stain typhoid flagella, even after the culture has grown for three or four days, it is more difficult to do so in such an old growth of *B. coli*. Even with a twenty to twenty-four hours old culture, the flagella of *B. coli* appear to be stained with greater difficulty than those of *B. typhosus*.

The hanging-drop shows that the bacilli hardly move at all, in most cases, beyond that rotatory swing, at times very rapid, known as 'Brownian movement.' This is seen also in the case of particles of cinnabar, or other finely divided inanimate object, when examined in a drop of water. There is, in these cases, no locomotion, or movement from place to place, across the field of the microscope.

Sometimes, however, locomotion is at first very rapid, and hardly to be distinguished from that of *B. typhosus*. This may be associated with the presence of a larger number of flagella than usual; but the rapidity of the motion dies away after a very short time, seldom lasting more than, at most, an hour or two. It has been stated that *B. coli* is altogether devoid of motility. This, however, is contrary to the general experience. If a hanging-drop be made from the fluid often present at the bottom of a glycerine-agar culture, the organisms may frequently be seen to dart about with the utmost rapidity, quite comparable with that seen in the case of *B. typhosus*. But the movements, as stated, soon become sluggish and then cease completely.

Sections of tissues containing typhoid bacilli

Bacillus typhosus in tissues.—The organism is best demonstrated in the spleen and mesenteric glands (fig. 96, A & B). From these, or from blood taken from the spleen, or from a rose spot during life, according to some accounts, pure cultures may be made. Since the introduction of Widal's sero-diagnosis test for typhoid fever, however, the German method of splenic puncture during life has been abandoned. In typhoid ulcers, and in tissues other than those mentioned, as also in the case of the urine, in which it is sometimes present, demonstration of the specific organism is not always easy.

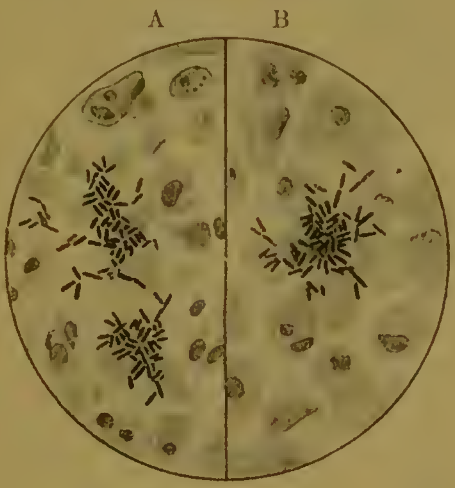


FIG. 96.—BACILLUS TYPHOSUS. × 925
A, section of spleen; B, section of mesenteric gland (Löffler's methylene blue).

Sections of spleen, or mesenteric gland, stained by Löffler's methylene-blue, and rapidly dehydrated by absolute alcohol, or better still, perhaps, by anilin oil, and then clarified in xylol, show the typhoid bacilli collected, here and there, into large and small groups, which by their intensely blue colour are readily detected, even under the low power of the microscope.

The isolation and differentiation of B. typhosus and B. coli

The use of media containing phenol, hydrochloric acid, and other antiseptics, such as the well-known 'Parietti broth,' and phenol gelatine (see pp. 5, 8), for the separation of *B. typhosus* and *B. coli*, is being more and more abandoned in favour of free dilution of the media. These antiseptics may be useful in checking the growth of associated non-pathogenic organisms liquefying gelatine. But Delépine, among others, relies entirely on the dilution method, both for the differentiation of the two bacilli under discussion, and for the choking off of troublesome associated organisms. He says,[1] 'By reducing the number of colonies obtained in a plate to five, ten, or twenty at most, I can watch the development of these colonies sufficiently long to separate the bacillus of typhoid fever from other bacilli. The advantage of this method is that the growth of the typhoid bacillus is much more rapid on ordinary alkaline media than on those to which antiseptic substances have been added for the purpose of checking the growth of other bacteria. On such alkaline media it is possible to obtain, in twenty-four hours, colonies of typhoid bacilli of such a size that it would take three or four days to grow on acid, carbolised, or iodised gelatine.'

To examine water suspected of contamination with the Bacillus typhosus or B. coli.—It is necessary in times of an epidemic of typhoid fever to examine the sources of contagion, which are most frequently water or milk, and the following is the usual method of procedure.

The water is collected in a sterile bottle,[2] and a large quantity must be examined before a negative opinion is given. The water is passed through a sterilised Pasteur-Chamberland filter, using either an exhaust apparatus connected with the main water supply, or an aspirator of some sort. With a sterile, handled nail-brush the 'candle' of the filter is carefully scrubbed down into a glass dish. (A sterile petri-dish containing sterile water may be employed; and peptone-water, or normal saline solution, may be used instead of plain water.) From the brushings thus obtained a series of cultivations on the usual media, especially 'shake' gelatine, or glucose agar, and plates of potato-gelatine and of glycerine agar-agar are made.

After incubating at the appropriate temperatures overnight, the 'shake' cultures will clearly demonstrate any *B. coli* present. Any colonies on the potato-gelatine or agar are examined with a low power of the microscope, and those resembling *B. typhosus* or *B. coli* are noted. From these, coverslip preparations are made, and if the appearance suggests one or other of these organisms, pure subcultures are made from the colony under observation, and the distinctive tests applied, as above mentioned.

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[1] Sheridan Delépine, 'On the Value of the different Bacteriological Methods of Diagnosis of Typhoid Fever,' *Med. Chron.*, March, 1899.

[2] For further details, consult p. 263 *et seq.*

Should the water be much contaminated, it is necessary to dilute freely before making the plates of potato-gelatine, as the colonies of *B. coli*, when numerous, have insufficient room to develop normally, and then are easily mistaken for young colonies of *B. typhosus*.

Typhoid sloughs.—In examining the sloughs which separate from typhoid ulcers of the intestine, the same general methods are used, great dilution before inoculation of media being necessary.

Inoculation experiments.—Cultures of typhoid bacilli soon lose their virulence unless this is maintained by passage through a series of animals, so that its inoculation into a susceptible animal like the guinea-pig or rabbit, whether subcutaneously, intraperitoneally, or intravenously, may be followed by no obvious effects.

To obtain a virulent culture of typhoid bacilli.—The general principle consists in lowering the vitality of the animal by means of injections of the chemical products of an ordinarily harmless bacillus, such as *B. subtilis*, *B. prodigiosus*, *B. coli*, &c., simultaneously with, or just before, the use of the culture of *B. typhosus*, which has lost its virulence. This is done in the case of a series of animals, using less and less of the *B. subtilis*, &c., in each of the successive animals; until, finally, the *B. typhosus* may be used alone to produce a fatal result.

Sanarelli's method, which is to be recommended, consists in injecting into the peritoneal cavity of the guinea-pig a broth culture of *B. coli*, which has been previously sterilised by heating to between 60° and 70° C. for ten minutes before injection. (It is as well to test whether the culture *has* been killed, by inoculating a broth tube therewith, and incubating it overnight at 37° C.) A half c.cm. of a twenty-four-hours-old broth culture of *B. typhosus* is then injected subcutaneously. Owing to the chemical products of the *B. coli* culture injected intraperitoneally, the guinea-pig will probably die in twenty-four hours or thereabouts, and care should be taken so that the animal may not die during the night—*i.e.* at a time when it is no longer under observation; if necessary, the animal may be killed shortly before the time at which death would otherwise result, so that cultures may be made from the peritoneal cavity before any transudation of *B. coli* has occurred from the intestine of the animal.

If cultivations made in this way contain a rod-shaped organism, it is probably *B. typhosus*, and not *B. coli*, cultivations in 'shake' gelatine, in milk, and on potato, together with flagella-staining, being used as tests. Lastly, the organism, which from such cultures, &c., appears to be *B. typhosus*, is then tested by being mixed with the blood of a patient suffering from typhoid fever.

If it is the typhoid bacillus, it should show a well-marked **Widal's serum reaction** (see p. 183).

A broth culture from the peritoneal cavity of the first inoculated animal being ascertained to be absolutely pure, and free from *B. coli*, the experiment is repeated with a second animal, using less of the sterilised coli culture intraperitoneally than in the first case, and injecting the new typhoid culture subcutaneously. This is repeated through a series of animals, using less and less, and finally none, of the coli culture. The purity of the new typhoid culture must be verified each time. Eventually, a high degree of virulence is obtained, but it requires to be constantly maintained '*per passage*.'

To obtain a pure culture of B. typhosus from a 'typhoid' spleen.—When removed intact from the body, it is washed in hot distilled water, and then with some antiseptic, such as absolute alcohol, or corrosive sublimate 1 in 1,000. With sterile knife the spleen is sectioned, and a culture made from the cut surface; or a little of the exposed spleen substance is placed in a flask containing 200 c.cm. of sterile water. After incubation at 37° C. for twenty-four hours, agar plates, already poured, are brushed over with sterile camel-hair brush dipped in the inoculated water. After incubation for twenty-four hours, a copious growth can be obtained, the virulence of cultures from a fresh spleen varying considerably.

The effects of inoculations with B. typhosus and B. coli

Bacillus typhosus.—*Intraperitoneal inoculation* of a guinea-pig with a small quantity of a virulent culture of *B. typhosus* causes death in less than twenty-four hours, with an exudation of highly toxic peritoneal fluid, from which a pure culture can be made. By the passage of intraperitoneal fluid thus obtained through a series of animals, the virulence can be much exalted. In cultures of low virulence the bacilli seem limited to the seat of inoculation, but with more virulent organisms pure growths can be obtained from the heart's blood, the spleen, and the liver.[1] (Occurrence in the blood is inconstant, and is denied by some authors.[2]) With still greater toxicity of the intraperitoneal exudation from a previous case, death followed intraperitoneal injection of 2 c.c. of the fluid in five and a half hours. After passage through a series of animals, 2 c.cm. of the intraperitoneal exudation caused death in five and a half hours after injection intraperitoneally; whilst 0·25 c.cm., in another experiment, injected intravenously, caused death in two and a half hours.

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[1] Professor Sidney Martin, F.R.S., 'The Chemical Products of Pathogenic Bacteria,' *Brit. Med. Journ.* 1898, i. p. 1645.

[2] Symmers, in Thoinot and Masselin's *Outlines of Bacteriology*. English edition, 1899.

Subcutaneous inoculation may cause local swelling and suppuration, a pure culture being obtained from the abscess, though the heart's blood, spleen, liver, and even peritoneum may be sterile. But general infection may result, especially if sterilised cultures of *B. coli*, &c., be injected intraperitoneally also. Enlargement and congestion of Peyer's patches have been described, and Rembinger stated [1] in 1897 that he had succeeded in producing this condition with commencing ulceration in the intestine, with a typical typhoid temperature chart, in the case of young rabbits fed on virulent cultures mixed with their food.

Bacillus coli.—*Intraperitoneal inoculation* of a virulent coli culture, derived from the spleen in the manner indicated on p. 182, frequently causes death in less than twenty-four hours, and it appears to be easier to increase the virulence, *per passage*, in the case of *B. coli* than in that of *B. typhosus*, or *Gärtner's bacillus*, to be referred to subsequently (see p. 189). The exudation into the peritoneal cavity in the cases of the typhoid and Gärtner bacilli is far less frequently hæmorrhagic than is the case with *B. coli*. The exudation becomes blood-stained from the presence of blood-colouring material, red corpuscles being absent (S. Martin).

After death, *B. coli* escapes from the intestine into the different organs of the body, and has distinctly putrefactive properties.

The exact nature of the poisonous agent, or agents, in the case of the typhoid and colon bacilli, is still undecided. A proteose has been isolated in the case of *B. typhosus*, having pathogenic properties, but ferment action has been suggested as being probable.

The serum diagnosis of typhoid fever

Widal's reaction. *Rapid clinical method.*—A broth subculture of *B. typhosus* is made from an agar culture of the same, which has been kept at the temperature of the laboratory for about three or four weeks. This is incubated at 37° C. overnight; the culture [2] should not be used when more than twenty-four hours old. Nine loopfuls of this broth are placed on a glass slide around one of the drops of blood, which has been obtained by pricking the finger-tip of the patient supposed to be suffering from typhoid fever. An emulsion is made by rubbing this blood up with the nine drops of broth, so that the former is diluted, roughly speaking, one in ten. Many prefer a dilution of 1 in 30, or more (see p. 189).

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[1] *Annales de l'Inst. Pasteur*, 1897.

[2] Instead of a broth culture, a very weak emulsion made by rubbing up a minute quantity of a one to three days' old agar culture with 10 c.c. distilled water, in a test-tube, may be used. Symmers (*loc. cit.*) makes the very useful suggestion that the broth culture, or agar emulsion, should be passed through wet filter paper, so as to obtain a fluid as free as possible from *clumps* of bacilli, which are probably largely responsible for some of the errors made by inexperienced observers.

A hanging-drop[1] is made by taking a loopful or two of this emulsion and placing it on the under surface of a coverslip, which is then placed over the depression in a hollow-ground slide in the usual way (p. 30).

A control experiment, which should always be made, is carried out in exactly the same way, using the blood of a normal individual, instead of that from the suspected case. The hanging-drops thus made are examined under the microscope (p. 31). It should first be ascertained, by looking at the control experiment, that the bacilli are actively motile, and show no marked tendency to collect together into groups all over the field.

The hanging-drop made with the suspected blood should show the following phenomena, if the case is one of typhoid fever. There is a great loss of motility, which may be obvious immediately the preparation is made, or it may take any time between half an hour and two hours; but, as a rule, this loss of motility should appear in under thirty minutes, and may be instantaneous. This loss of motility is further associated with the massing together of the bacilli, which form little groups all over the field, and in most of the fields that can be examined. This grouping together, or clumping, of the bacilli is known as '**agglutination**,' the bacilli sticking together to form a felt-work, the individuals of which are absolutely motionless, or show only slight Brownian movement.

It is absolutely essential to make the control test with healthy blood, and to watch it from time to time to make sure that the broth culture, or emulsion, is acting normally. If this culture be made exactly as directed, and the blood diluted to the extent indicated, the vast majority of observations made throughout the civilised world tend to prove that no disease but typhoid fever can produce Widal's reaction with a culture of *B. typhosus* (see, however, p. 189).

Widal's original method, which is a slower process, consists in mixing the serum, which has been separated from the blood of the typhoid patient, with nutrient broth in the proportion of one of serum to ten or fifteen parts of broth. After inoculating this mixture with a culture of *B. typhosus*, it is incubated at 37° C., and in twenty-four hours' time a precipitate forms at the bottom of the tube, the supernatant broth being quite clear. Should the patient not be suffering from typhoid fever, the serum remains uniformly turbid after twenty-four hours, a cloud settling at the bottom only after some days.

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[1] Some writers prefer to use a coverslip and plain slide, rather than the hanging-drop method.

In 1896 Widal showed that the reaction, which now goes by his name, occurs in an equally characteristic manner, as regards agglomeration of bacilli, whether the culture of *B. typhosus* is living or dead (killed by an exposure to a temperature of 60° C. for ten minutes).

This observation was confirmed, and extended to the organism of Malta fever—the *Micrococcus Melitensis* of Bruce—by Wright and Semple, of Netley. They point out [1] the great advantages of thus being able to use dead cultures, which may be safely and conveniently carried about, and sent everywhere. An incubator and a supply of culture media are, therefore, no longer necessary for practitioners in remote districts wishing to make use of Widal's reaction.

As further facilitating matters, Wright and Semple strongly recommend the use of **capillary sero-sedimentation tubes**, and a modification [2] of Widal's long method already mentioned. The use of these tubes (which can be made by any one in a few moments from ordinary glass tubing) does away with the need for prolonged microscopic observations, and may, where the necessity arises, be employed even in the absence of a microscope. A further advantage of using sedimentation tubes is that it 'lends itself better than any other method to the quantitative determination of the intensity of the serum reaction.'

Wright and Semple's method.[2]—A few lengths of glass tubing, with which to make 'blood capsules' and sealed sedimentation tubes, a dead culture of typhoid bacilli, a supply of normal salt solution, a watch-glass or two, and a blowpipe flame [3] are all that are necessary for carrying out the sero-diagnosis reaction.

The technique for the manufacture of the tubes is as follows:—

(i) *Blood capsules.*—Heat a narrow piece of glass tubing, in two places in succession, and draw it out at these places into capillary tubes, leaving the intervening piece to form a little chamber, or 'blood capsule.' The piece of tubing at this stage somewhat resembles in shape the common fishing-float, spindle-shaped in the centre and tapering at its extremities. One of the capillary tubes (which will be the lower one, finally) is bent up so as to form a somewhat acute angle with the long axis of the capsule. To avoid a kink at the bend, the capillary tube when first made should be allowed time to cool down, and it is then bent over very slowly.

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[1] Wright and Semple, 'On the Employment of Dead Bacteria in the Serum Diagnosis of Typhoid and Malta Fever,' *Brit. Med Journ.* 1897, i. p. 1214.

[2] Wright, 'A Further Note on the Technique of Serum Diagnosis,' *loc. cit.* 1898, i. pp. 355–357. After one or two trials, this method, which is extremely simple. really, can be carried out very rapidly.

[3] As a makeshift. the authors suggest an alcoholic spray, which by ignition can be converted into a blowpipe flame.

(ii) *Sealed sedimentation tubes.* These are practically of the same shape and make [1] as the glass pipette shown in fig. 62, p. 120, except that the mouthpiece at the upper end of the pipette (which, of course, is not plugged with wool) is fused, and drawn out into a fine point. We have thus a pipette closed at its upper end and open at the lower extremity of the terminal, fine capillary tube. The bulb nearer this lower extremity (fig. 62) in this sedimentation tube, which we have supposed to have been extemporised from a pipette, is called the 'mixing chamber;' whilst the fused upper extremity, or closed mouthpiece of the pipette, is called the 'air chamber.'

Method.—*a*, *The blood-capsule is half-filled* with blood from the cleansed finger of the patient, by applying the bent-up free end of the lower capillary tube to the bleeding point. Capillary attraction and gravity cause the blood to flow in, and, when the lower half of the blood-chamber or capsule is full, the upper half is rapidly warmed with a match-light or spirit-lamp flame. The air in the upper half is rarefied, and before it can contract again the tip of the upper capillary tube is sealed in the flame. When the capsule cools, the blood is drawn up into the capsule from the lower bent capillary tube, and its tip fused without any danger of overheating the blood.

β, *Dilution of the serum.*—In a short time the serum separates from the blood, and, after the upper capillary tube has been broken off just above the capsule, it is to be aspirated into the sealed sedimentation tube described above. The 'air chamber' at the upper end of this tube is heated, and the free lower extremity is passed into the blood capsule. Before doing so, however, a mark on the capillary tube below the 'mixing chamber' at, say, ¾ inch from its tip, should be made with a wax pencil.

γ, *Mixture of the serum with bacterial culture.*—As soon as the serum runs up to this mark, the sedimentation tube is withdrawn from the blood capsule and its tip introduced into a watch-glass of normal saline solution. A bubble of air will have entered the tip of the tube before it reaches the watch-glass, and it is used as an index. The salt solution is allowed to run up into the pipette until the lower, or distal, end of the air-bubble index reaches the wax pencil mark. This means that salt solution, exactly equal in volume to that of the serum, has entered the tube. The point of the tube is momentarily raised out of the salt solution, so as to allow another air-bubble to enter and form a second index. The tip of the tube is once more lowered into the solution. In this way the sedimentation tube can rapidly be filled with any desired number of equal volumes of salt solution.

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[1] Further details and illustrations are given in the *Brit. Med. Journ.* for 1898, quoted above. Blood capsules and sedimentation tubes can be obtained, ready made, from Mr. A. E. Dean, 73 Hatton Garden, E.C. The upper end of the 'air chamber' described need not be closed, filling of the mixing chamber being effected by an ingenious mechanical appliance devised by Mr. Dean, consisting of a short metal barrel, fitting on to the upper end of the air chamber, and a rubber teat compressible directly, and through a spring worked by a screw.

δ. **For ordinary serum diagnosis**, where a tenfold dilution is to be employed, one volume of serum (*i.e.* up to the mark), and then four equal volumes of salt solution, are introduced into the sedimentation tube. The tip of the pipette is then transferred to the bacterial emulsion with which the serum is to be tested (see below). Five equal volumes of this emulsion are then passed up into the pipette, which thus contains one volume serum, four volumes saline solution, and five volumes bacterial emulsion—*i.e.* the serum is diluted to 1 in 10.

ε. *Mixture of the contents of the sedimentation tube* is readily effected by allowing the air in the 'air chamber' to draw up the whole fluid in the 'mixing chamber.' If sufficient aspiratory force has been obtained at the outset, this will effect itself automatically. If, on the other hand, sufficient aspiratory force was not then obtained, it will be necessary to break off the upper fused end of the air chamber. The air in this chamber may then be reheated without displacing the contents of the tube. As each successive volume of fluid is drawn up into the air chamber its air-bubble index rises to the surface and escapes. To facilitate this, the tubing should be fairly wide, and the mixing chamber large—about one inch long. Mixture being effected, gentle heat is again applied to the air chamber, and the fluid driven down into the capillary tube below the mixing chamber. The tip of this tube is quickly sealed in the flame. The segmentation tube is placed upright (tip downwards) in a test-tube, which is labelled with the date, the patient's name, and the dilution of the serum.

A control tube containing a mixture of equal volumes of bacterial culture and normal saline solution should always be made.

When it is desired to make a quantitative estimation of the intensity of the serum reaction, the method is as follows: Introduce one volume of serum into the pipette as before. Secondly, take in four, or more, equal volumes of normal saline solution. Thirdly, expel the diluted serum into a clean watch-glass. Fourthly, mix one volume of the diluted serum, or of any further dilution that may have been made from it, with one volume of the bacterial culture.

Dead bacterial cultures are the most convenient form to employ in these sero-sedimentation tubes. Young agar cultures of the desired micro-organism are emulsified with a measured quantity of sterile normal salt solution—4 or 5 c.c. of salt solution to each agar tube. The emulsions are killed by heating to 60° C. for ten minutes. An addition of 0·5 per cent. carbolic acid is added to the dead cultures in order to prevent the possibility of accidental contamination. The dead and carbolised cultures are filled in from a syringe into small capsules, or where large quantities are required, for class purposes, &c., the dead cultures are kept in drop bottles fitted with pipette, over the blown-out open end of which is stretched a thin piece of rubber.

The results obtained by this method.—The positive or negative result of a blood examination in an ordinary case can be judged of in the course of twelve hours by a mere naked-eye inspection of the sedimentation tubes.

With a negative result, the fluid remains turbid throughout.

A positive result is indicated by an unevenly disposed, flocculent precipitate, consisting of masses of agglomerated bacilli settling at the closed lower extremity of the pipette, whilst the supernatant fluid becomes quite clear. A less complete positive reaction is indicated by the deposit of a flocculent precipitate as before, whilst the supernatant fluid is incompletely clarified.

In a control tube there is eventually a deposit of evenly stratified, non-flocculent precipitate, which is readily distinguished from that seen in a positive reaction.

The use of serum free from blood corpuscles, and the employment of salt solution instead of distilled water, prevent ambiguity in the appearance of the deposit, which might arise from the mixture of the deposit in the control with red corpuscles, or the occurrence of a sediment of flakes of serum-globulin.

The microscopic examination of the sediment reveals agglomerated bacilli in the positive result, and non-agglomerated bacilli in the control. By such examination of the fluid at the bottom of the pipette one may, in fact, determine whether the reaction is positive or negative, in a few minutes from the time the test has been commenced.

For this purpose, if a sealed sedimentation tube has been used, the fused upper end should first be nipped off, and then the tip.

In conclusion, Professor Wright notes that the sedimentation tubes may be used many times. They may be blown out after breaking off the ends, cleaned in distilled water, and dried by passing through the flame.

After more than three years' most careful investigation of this reaction, throughout the civilised world, the blood or blood-serum from, practically, all known kinds of disease having failed to give the reaction with a properly prepared culture of *B. typhosus* unless the case proved to be enteric fever, it is now fair to assume that Widal's serum-diagnosis method is reliable; and even negative results, after the fifth day of the symptoms (with all the above-mentioned precautions, the healthy blood, in the control experiment, failing to cause agglomeration), may be taken as positive proof of the absence of typhoid fever. In four hundred hospital cases reported in September, 1897, at the Leeds Congress of the Sanitary Institute,[1] the serum diagnosis was supported by the course observed clinically, and all post-mortems of fatal cases confirmed the result of the test. In general practice, also, the medical attendants testified to the correctness of both negative and positive results in not less than 94 per cent. of them.

Bacillus coli.—*Widal's reaction* can also be demonstrated in the case of *B. coli*, when artificially prepared coli serum is prepared from an animal immunised, by gradually increasing injections of *B. coli* cultures, in the usual way. It has been stated, however, that normal rabbit serum gives a clumping reaction with *B. coli*. The reaction does not occur with all coli serums, some serums only agglutinating certain coli cultures. This points conclusively to there being several varieties at least of this organism. Variations in the degree of indol reaction and milk coagulation point in the same direction.

Widal's reaction is specific in most cases; that is to say, it is not given when blood serum from a case of enteric fever is mixed with

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[1] *Brit. Med. Journ.* December 4, 1897.

B. coli; nor does coli serum give a reaction with *B. typhosus*. According to Durham, however, the *Bacillus enteritidis* of Gärtner, which in many ways resembles both *B. coli* and *B. typhosus*, gives the reaction to some extent, when mixed with typhoid serum; and if one drop of the blood serum from a patient suffering infection with Gärtner's bacillus (from eating unsound meat,) be mixed with nine drops of typhoid broth, a positive reaction—*i.e.* clumping of the bacilli—occurs, proving that the dilution of 1 in 10 is too small to furnish a reliable criterion. With a dilution of 1 in 100, the reaction is negative (Durham).

Inoculation against enteric fever, with serum from an immunised horse, has been extensively practised by Wright,[1] as a preventive measure, with apparently good results. The method is still under trial.

Bacillus enteritidis of Gärtner

Of recent years the attention of bacteriologists has been drawn to the many serious, and often rapidly fatal, cases of illness arising from eating unsound meat, especially when uncooked, or when not eaten for some days after being cooked. Many of these have been attributed to the presence of sterile chemical products of bacterial activity, in the absence of living germs. The majority of cases which have been investigated bacteriologically have, however, been shown to be due to **Gärtner's bacillus**, of which there appear to be several varieties.

For an account of some of these epidemics, recent papers by Durham[2] should be consulted. In one instance,[3] of ninety-three persons who partook of the meat, fifty-eight became ill, and one died thirty-six hours later. The symptoms include nausea, vomiting, diarrhœa, weakness, prostration, fever, and pains in the joints. The organism is found in the diseased meat, distending the capillaries, and in the viscera of the patient after death. Puerperal trouble in cows, and septicæmic diarrhœa in calves, have frequently been known to have affected the animals conveying disease in this way. Gärtner's bacillus is of interest, not only because the results of inoculation are similar to those produced by *B. typhosus* and *B. coli*, but on account of its morphological and cultural peculiarities, which closely resemble those of the two organisms mentioned.

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[1] *Brit. Med. Journ.* 1899, i. p. 572.

[2] Durham, 'On the Present Knowledge of Outbreaks due to Meat Poisoning,' *Brit. Med. Journ.* 1898, i. p. 1797.

[3] *Ibid.* 'On Infections by Unsound Meat, more especially with regard to *B. enteritidis* (Gärtner),' *Trans. Path. Soc.* 1899, and *Brit. Med. Journ.* 1899, i. p. 1216.

Durham[1] gives the following description :—

Microscopic characters of B. enteritidis (Gärtner).—1. *Young agar cultures*, polymorphous. Threads, bacilli of various lengths, and round, coccus-like bodies (similar to typhoid bacillus).

2. *Gelatine streak cultures*, a few days old, may show the characteristic forms, the extremities of which ends do not stain, as noted by Gärtner ; in recently divided individuals, the stained material is all at one end. This appearance is not always obtained unless the culture is of the right age.

Cultivations.—Growth in all media is, like that of *B. coli*, much more exuberant than in the case of *B. typhosus*.

The chief points of difference between the three organisms referred to may be tabulated as follows :—

	B. typhosus	**B. coli communis**	**B. enteritidis** (Gärtner)
Glucose gelatine, and glucose agar	No gas formation.	Gas formation.	Gas formation.
Potato . .	Growth hardly visible, forms a 'transparent glaze.'	Growth fleshy, and yellowish-brown.	Slightly coloured growth.
Milk . .	No coagulation.	Coagulation (generally).	No coagulation.
Indol reaction	Absent.	Present (generally).	Only a slight reaction, not constantly obtained.
Flagella . .	Long, wavy, and numerous, eight to sixteen or twenty	Shorter, and less numerous, two to eight or ten. Stained with more difficulty than those of *B. typhosus*.	About intermediate between *B. coli* and *B typhosus*.
Widal's reaction	With serum from typhoid patient, not with coli serum.	Not with serum from typhoid patient, only with coli serum.	With Gärtner serum, and with typhoid serum, if dilution be 1 in 10, not when 1 in 100 (Durham).

Widal's reaction, referred to in the table, has been used by Durham as a means of diagnosis in cases of infection from unsound meat. blood serum from patients even eight days after the onset of symptoms giving a positive result, when diluted 1 in 100, and mixed with a culture of *B. enteritidis* (*Gärtner*).

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[1] Durham, 'On the Present Knowledge of Outbreaks due to Meat Poisoning,' *Brit. Med. Journ.* 1898. i. p. 1797.

Inoculation of guinea-pigs, intraperitoneally, with a small loopful of a virulent culture causes death in less than twenty-four hours, with exudation of a highly toxic,[1] albuminous, but not hæmorrhagic fluid, teeming with the specific organisms, which are also found throughout the organs of the body, its distribution in these being much more constant than in the case of *B. typhosus*. It occurs in the heart's blood, the spleen, liver, kidneys, and peritoneal exudation, in pure culture.

Micrococcus Melitensis, and Malta fever

Micrococcus Melitensis, the organism of Malta, or Mediterranean, fever (endemic in various parts of the Mediterranean, and possibly in the Red Sea, Hong Kong, and elsewhere), was first described in 1886 by Bruce, and valuable work was also done[2] by the late Captain Louis Hughes, R.A.M.C., in 1896, and also by Wright and Semple.[3] The micrococci, which are extremely small, were isolated from the enlarged and softened spleen, which is such a marked feature in the disease. When inoculated into monkeys, Koch's postulates are fulfilled by the organism, the peculiarly irregular temperature curve, consisting of intermittent waves of pyrexia of a distinctly remittent type, characteristic of the disease in man, being reproduced.

The absence of ulceration in the intestines, as well as the clinical course, distinguish the disease from typhoid fever. The blood, spleen, &c., have been shown free from malarial parasites and pigment.

Cultivation.—The organism is best grown on agar, appearing, in from four to five days, as minute transparent colonies; subsequently they become opaque and of an orange tint (Hewlett).

Film preparations should be stained with carbol-fuchsin. The cocci are seen to be arranged singly, or in pairs, or in short chains. They are decolorised when treated by Gram's method.

Widal's reaction can be readily demonstrated by mixing the blood-serum from a patient suffering from the disease with a pure culture of the specific organism, alive or dead, after diluting exactly as in the case of *B. typhosus*, &c.

The disease is most prevalent during the hot dry months, between May and October, with a maximum prevalence in July, August, and September, occurring in inverse ratio to the amount and continuance of the rainfall. The disease, which is not contagious, appears to spread by aërial infection, rising from drains, or soil infected by the excrement of previous sufferers from the disease, and so entering the system by the throat or air passages. The mortality does not exceed 2 per cent., but the duration of the sickness is very long (Hughes).

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[1] Professor Sidney Martin, F.R.S., Croonian Lectures, *Brit. Med. Journ.* 1898, ii. p. 13.

[2] Captain M. Louis Hughes, R.A.M.C., 'The Endemic Fever of the Mediterranean,' *Brit. Med. Journ.* 1896, i. p. 972.

[3] 'On the Employment of Dead Bacteria in the Serum Diagnosis of Typhoid and Malta Fever,' *Brit. Med. Journ.* 1897, i. p. 1214.

Bacillus icteroides and Yellow Fever.—**Yellow Fever**, as shown[1] by Sanarelli in 1897, is a disease taking a cyclical course, and it is solely at the end of the cycle, about the seventh or eighth day, that the specific microbe (**B. icteroides**), at first present in very small numbers, multiplies rapidly, and invades, almost suddenly, the whole organism, being generally accompanied by other microbes—probably from the intestines. The manner of development of these secondary organisms gives yellow fever its protean character.

Only in cases which regularly complete the disease cycle can the specific microbe, diffused in the blood and organs, be found with comparative facility. When an intervening septicæmia, or precocious uræmic poisoning, puts an early end to the disease cycle, it is extremely difficult, if not impossible, to isolate the *B. icteroides*.

The secondary infections are nearly always due to *B. coli*, *streptococci*, *staphylococci*, and similar well-defined microbes; and, as in the case of typhoid, yellow fever is associated with quite a remarkable increase in the number of *B. coli*, which are met with in a state of almost absolute purity (see p. 172).

On the other hand, *B. icteroides* is *never found in the gastro-intestinal canal*, and must be sought for in the blood and tissues, where it is found in the capillaries of the liver, kidneys, &c., aggregated in groups. A fresh piece of diseased liver should be kept for twenty-four hours in the incubator at 37° C., as this facilitates the multiplication, and so the detection, of the organism.

Cultivations.—All the ordinary nutritive media may be used. The organism is a facultative anaërobe (see p. 36).

Gelatine plates.—The rounded colonies, at first transparent and granular, acquire an opaque nucleus, then become more granular, and opaque, milky-white, throughout. The gelatine is not liquefied. Growth on this medium seems to be facilitated if moulds are present.

Agar agar.—The culture should be kept for from twelve to sixteen hours at 37° C., and then at the room temperature for the same length of time. It then shows a flat central nucleus, transparent and bluish, surrounded by a prominent and opaque zone, the whole resembling a drop of sealing wax.

As this character, which for the present may be considered specific, can, fortunately, be obtained even in twenty-four hours, it serves to establish in the most rapid and certain manner the bacteriological diagnosis of *B. icteroides*.

The bacillus resists drying, dies in water at 60° C., and is killed in seven hours by the solar rays, but lives for a long time in sea-water.

Under the microscope, the bacillus is seen to be a rod with rounded extremities, and occurs generally united in pairs, in the culture; and in small groups, in the capillaries of tissues affected. It measures from two to four thousandths of a millimetre in length, and, as a rule, is two or three times longer than broad. It is stained by Gram's method.

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[1] This account is taken from Sanarelli's paper translated in the *Brit. Med. Journal*, July 3, 1897.

LESSON XVIII

CHOLERA—KOCH'S COMMA BACILLUS AND ALLIED ORGANISMS

A. **Koch's comma bacillus (Vibrio choleræ asiaticæ)**
B. **Vibrio Metchnikovi**
C. **Vibrio Finkler-Prior**

I. *Make cultivations* of A, B, and C:
  (i) Broth.
  (ii) Peptone-water.
  (iii) Gelatine stab.
  (iv) Gelatine plates. Examine and sketch, daily, under $\frac{2}{3}$-inch objective.
  (v) Agar streak. Keep at 20°-22° C.
  (vi) Potato.

II. *Stain coverslip preparations* of A, B, and C, from the agar culture (kept at 20°–22° C. for not more than twenty-four hours), with carbol-fuchsin.

III. *Make hanging-drops* from A, B, and C broths.

IV. Do the indol reaction with the peptone-water cultures, after a few hours' incubation, by adding a few drops of concentrated sulphuric acid, the nitrite solution added in the case of *B. coli communis* (p. 169) being unnecessary.

V. *Make impression preparations* from the gelatine plates, when the colonies are twenty-four hours old, and stain with carbol-fuchsin.

The bacillus of Asiatic cholera, Vibrio choleræ asiaticæ (Koch's comma bacillus)

The bacillus of Asiatic cholera.—This organism, which Professor Koch, of Berlin, has shown to be invariably associated with cases of Asiatic cholera, presents the following appearances.

Cultivations.—*Gelatine stab.*—In the first twenty-four hours the growth is hardly perceptible (fig. 97, A). In from thirty-six to forty-eight hours at 22° C., a slight liquefaction, with excavation of the gelatine from evaporation, is seen to have occurred at the top of the needle track, below which appears an irregular, opaque white line, composed of minute colonies of the organism (fig. 98, A). This white growth continues to spread downwards along the track of the needle, the liquefaction steadily increases, and with it the size of the excavated cup, from the evaporation of the supernatant fluid, the orifice of this cup in the gelatine being somewhat smaller than the

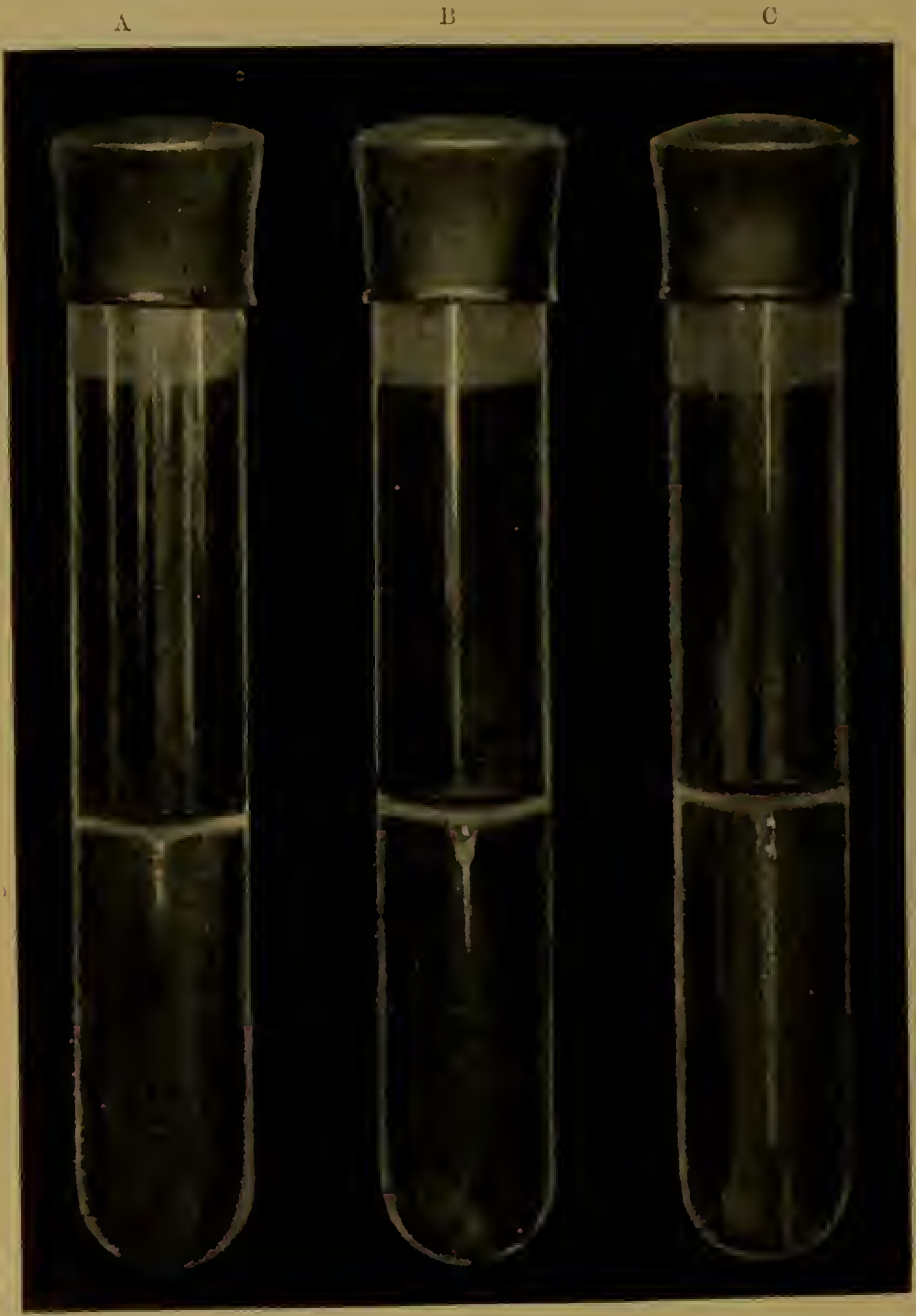

Fig. 97. A, VIBRIO CHOLERÆ ASIATICÆ. B, VIBRIO METCHNIKOVI. C, VIBRIO FINKLER-PRIOR

Stab-gelatine cultures, after twenty one hours.

gas-bubble itself in diameter. On the third day, after incubation at 22° C. (fig. 99, A), there is seen to be considerable increase in the development of the down-growth of opaque white colonies in the needle track, liquefaction also becoming increasingly evident.

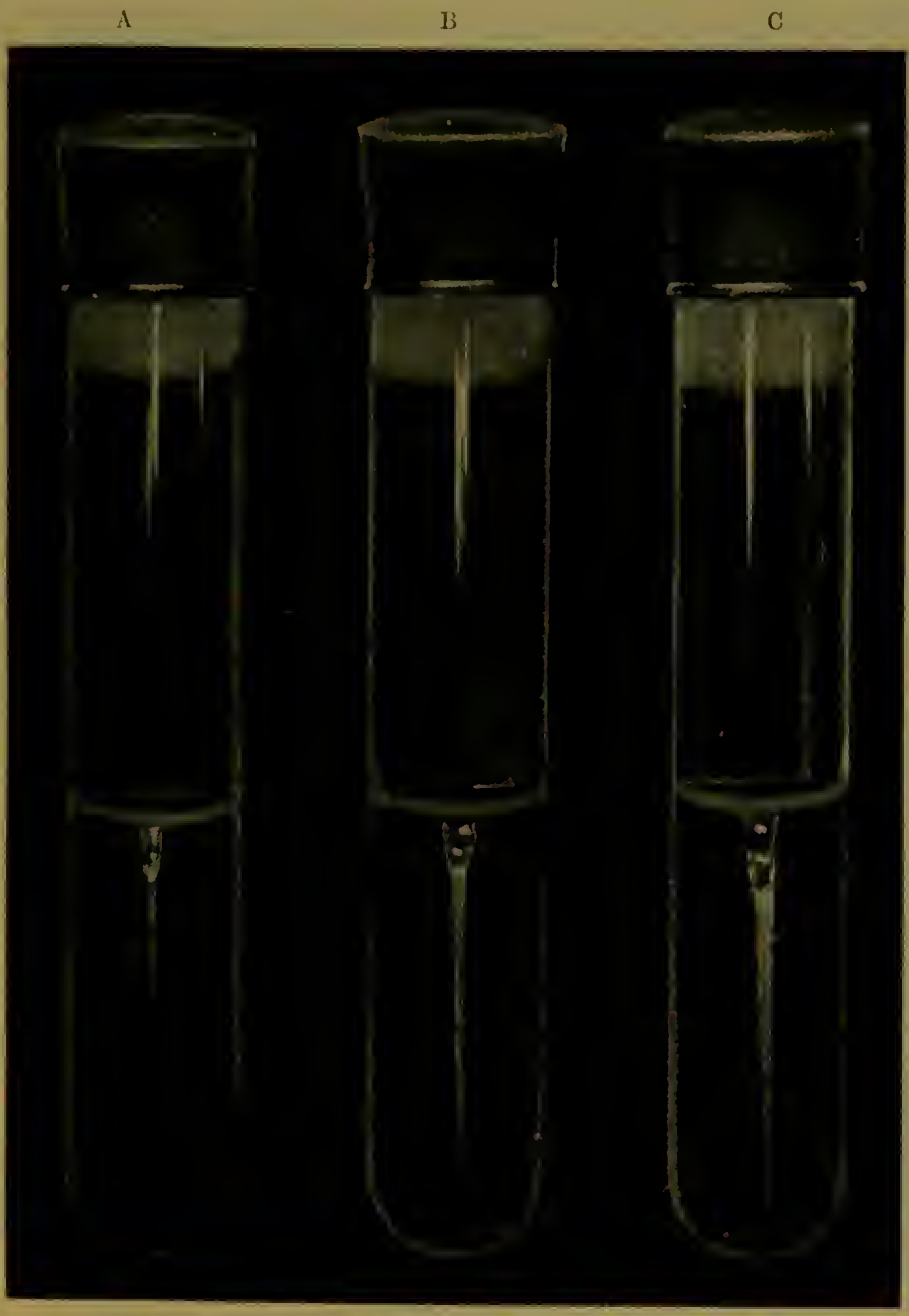

Fig. 98.—A, vibrio choleræ asiaticæ. B, vibrio metchnikovi. C, vibrio finkler-prior

Stab-gelatine cultures, after forty-five hours.

Plates.—Plates in from twelve to sixteen, or eighteen hours begin to show, under the microscope, minute points of growth. In about

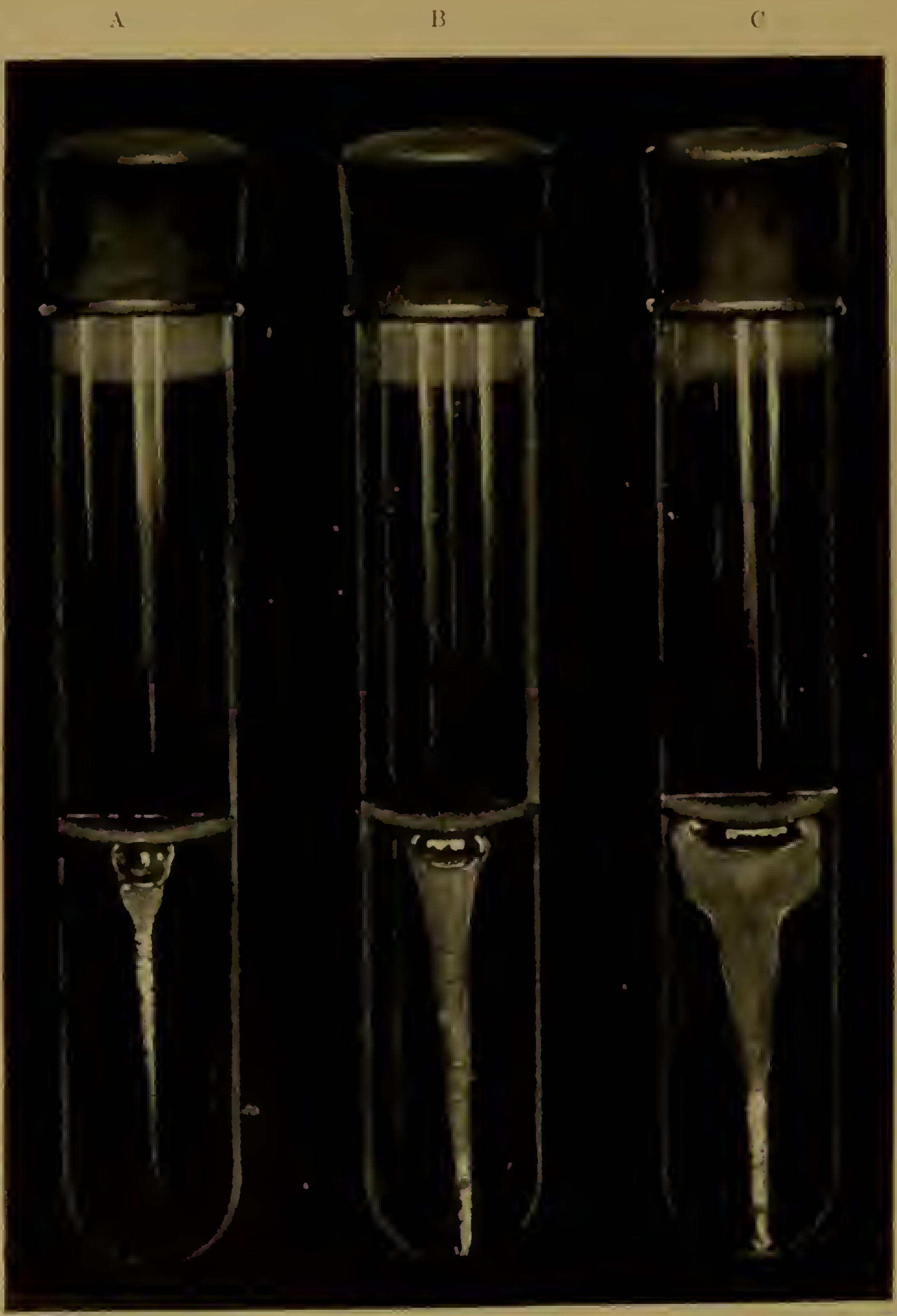

Fig. 99.—A, VIBRIO CHOLERÆ ASIATICÆ. B, VIBRIO METCHNIKOVI. C, VIBRIO FINKLER-PRIOR

Stab gelatine cultures, after ninety-three hours.

twenty-four hours the 'original' plate is so crowded with colonies as to present the appearance of ground glass.

Microscopic examination.—Young colonies, sixteen to eighteen hours old, are of very pale greenish-yellow tint, translucent, and sharply

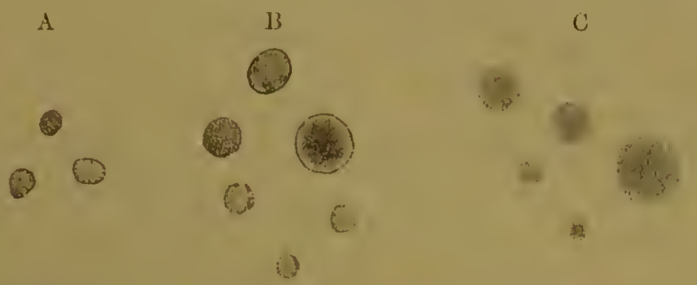

FIG. 100.—A, VIBRIO CHOLERÆ ASIATICÆ; B, VIBRIO METCHNIKOVI; C, VIBRIO FINKLER-PRIOR

Gelatine plate cultures, the first day (about twenty-four hours) after incubation at 22° C.

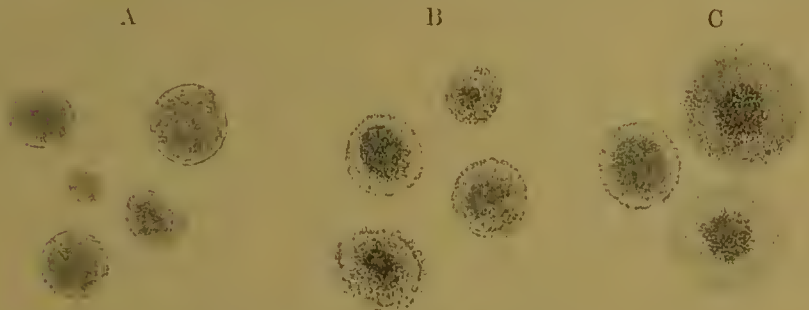

FIG. 101.—A, VIBRIO CHOLERÆ ASIATICÆ; B, VIBRIO METCHNIKOVI; C, VIBRIO FINKLER-PRIOR

Gelatine plate cultures, the second day (about forty-eight hours) after incubation at 22° C.

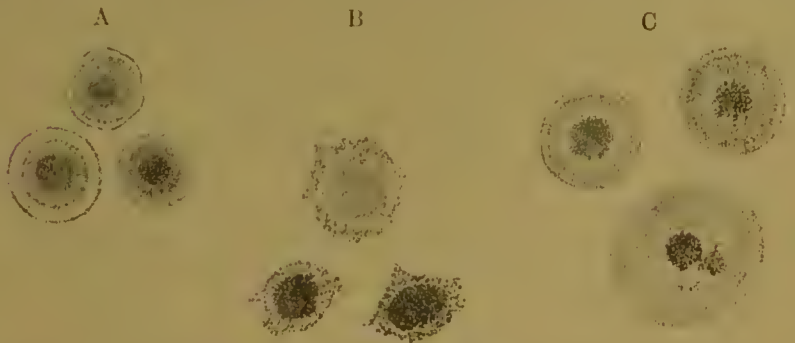

FIG. 102.—A, VIBRIO CHOLERÆ ASIATICÆ; B, VIBRIO METCHNIKOVI; C, VIBRIO FINKLER-PRIOR

Gelatine plate cultures, the third day (about sixty hours) after incubation at 22° C.

outlined. They are more or less homogeneous. In from twenty-four to twenty-six hours they lose this structureless appearance and become more granular (fig. 100, A), and, later, become even slightly lobulated, the margin of the colony being irregular, owing to the liquefaction of the gelatine. After forty-eight hours, or less, the aspect becomes still more characteristic, owing to the increasing liquefaction of the gelatine. The darker central portion is ragged and irregular at its edges, lying apparently in an almost clear space, which is really liquefied medium, to the bottom of which the remains of the colony, now yellowish-white, and rather opaque, has sunk. This clear space is limited by a sharply defined margin. The double contour thus presented is very striking. In from sixty-four to seventy-two hours, the central colony forms an islet with granular surface and very irregular edges, which radiate into the liquefying medium, the size of the colony being about three times that at forty-eight hours. This broken-down, granular-looking mass becomes fainter and fainter, until the whole surface of the plate is completely liquefied.

Agar-agar streak.—The growth is moist and greyish white, but not characteristic. The organism shows great tendency to involute when the culture is incubated at 37° C. A twenty-four hours' growth, best kept at from 20° to 22° C., is, therefore, very useful when one wishes to obtain non-involuted forms of the organism.

Potato.—The growth in twenty-four hours is of a light yellow colour, and hardly perceptible.

The cholera-red, or indol reaction, obtained by adding a few drops of pure sulphuric acid to an eight to twelve hours' culture of cholera bacilli in peptone-water (*without the use of any nitrite* solution, as in the case of *B. coli*), is very characteristic. A distinct reaction is obtainable after even a few hours' incubation. If the peptone-water growth is two or three days old, the crimson tint is still more pronounced. It depends upon the rapid formation of indol from proteid material, and the reduction of nitrates to nitrites (which then combine with the indol), as the result of the vital activity of the cholera organism, peptone-water being a more favourable medium than nutrient broth for the demonstration of this *nitroso-indol*, or *cholera-red* reaction.

Under the microscope, the cholera organisms are seen to be slightly curved, slender rods, with pointed ends, very like the German comma (*not* the English). The length is from half to two-thirds that of an average tubercle bacillus—*i.e.* one-eighth to one-third of the diameter of a red blood corpuscle. These commata are joined up with their concavities in the same direction, or, what is perhaps more common, with their concavities reversed, so as to produce an **S**-shaped figure. Three or more organisms, with their concavities in opposite directions, may thus be linked together, forming a **spirillum**; in fact, Koch has considered it possible that the cholera **vibrio** is really derived from a spirillum which has split up into its component parts.

In an ordinary film preparation, unless it has been very lightly spread out by means of the platinum loop, there may be no obvious grouping of the organisms visible; but in impression preparations (fig. 103), or when a flake of the characteristic 'rice-water stool' (fig. 104) is carefully spread on the coverslip and stained, the bacilli are often seen arranged in groups, individuals of each group lying parallel with each other, though the groups themselves may be arranged in various directions, but, viewed as a whole, producing an appearance which Koch, long ago, compared with 'schools of fish swimming up stream.'

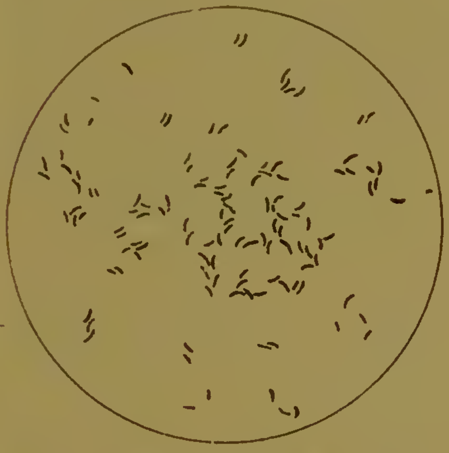

FIG. 103. VIBRIO CHOLERÆ ASIATICÆ × 530

Agar-agar plate, three days old, kept at 20°–22° C. Impression preparation

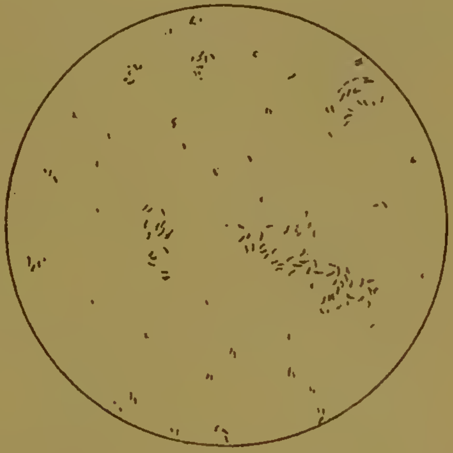

FIG. 104.—A FILM MADE DIRECT FROM CHOLERA 'RICE-WATER STOOL,' FORMING, REALLY, ALMOST A PURE CULTURE OF KOCH'S COMMA BACILLUS. × 530

This specimen was prepared by the author in Professor Koch's laboratory during an epidemic of cholera. The cultures, subsequently made, were typical in every way.

In the rice-water preparation (fig. 104), the commata are seen to be smaller and thinner than in preparations taken from cultures. The organisms are not stained by Gram's method.

In a hanging-drop, the vibrios can be seen to be very active, darting across the field very rapidly. This movement is due to the presence of a single flagellum, about one to one and a half times the length of the bacillus. It may be demonstrated by Van Ermengem's method (p. 171).

To examine water, suspected of being the source of infection, or **the dejecta, from a case of supposed cholera**, the following methods are in general use:

Suspected water, collected in sterile bottles, and carefully sealed up and rapidly brought to the laboratory, is placed in a series of small, sterile, conical Erlenmeyer's flasks, and to every 100 c.c. of water are added 1 gramme of peptone, ½ gramme of common salt, as recommended by Dunham, and thus making '*Dunham's solution* ;' or, in addition, 2 grammes of gelatine may be used, according to *Metchnikoff's* formula. After incubation at 37° C. for eight hours, the cholera bacilli, if present, will have multiplied more rapidly than other organisms.

Film preparations from the surface of this fluid, near its edge, may reveal a practically pure culture of comma bacilli. If so, pure cultures should at once be made—stab-gelatine, streak-agar, gelatine and agar plates, and peptone-water. The agar plates, kept at 37° C., will be ready for examination in, if need be, ten hours, and the peptone-water, in even fewer hours, ought to give a distinct cholera-red reaction, if cholera is present.

If this reaction is given, an ordinary loopful of the agar culture is injected into the peritoneal cavity of a guinea-pig, which should not survive more than a few hours, if the culture is that of cholera.

If the guinea-pig dies, one more test—Pfeiffer's reaction—is tried.

Pfeiffer's reaction.—This, as originally carried out, consists in mixing in a test-tube a standard loopful of the supposed cholera culture with a definite quantity of serum from an animal previously immunised against cholera. This is injected into the peritoneal cavity of a fresh guinea-pig. After twenty minutes some of the peritoneal fluid is drawn off in a pipette and a hanging-drop made from it. If the bacilli are found actively motile and unaltered in form, it proves that they were not the cholera vibrio. If they are found motionless and their outlines altered, so that the bacilli are almost spherical, then we may conclude that they are really cholera organisms, rendered motionless, as in Widal's reaction—discovered several years later—for the sero-diagnosis of typhoid fever.

Durham-Gruber Reaction.[1]—To ascertain the specific nature of the comma bacillus—which has been isolated and then shown to be pathogenic—the serum of an animal immunised against cholera, diluted 1 in 10, is mixed with an emulsion of the suspected bacillus, and poured into a narrow pointed test-tube made by fusing and drawing out a piece of glass tubing. The mixed culture and serum is incubated at 37° C., for an hour. If the comma bacilli are those of cholera, they will be found at the bottom of the tube, aggregated together as a white deposit, the supernatant fluid being clear; if otherwise, the fluid remains turbid, no special deposit being noticeable at the bottom. The Durham-Gruber reaction is similarly obtained, also, in the case of *B. typhosus*, *B. coli*, *Micrococcus Melitensis*, &c.

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[1] This has largely replaced the, often, less convenient method of Pfeiffer, which, however, is still extremely useful as a means of distinction between the various vibrios resembling Koch's comma bacillus.

A hanging-drop, prepared from the diluted serum and suspected culture, should show the bacilli motionless and aggregated into clumps, if true cholera bacilli.

Widal's reaction can be demonstrated by mixing the diluted blood-serum of a patient suffering from cholera with a young culture cholera bacilli, as directed in the case of typhoid fever.

The hanging-drop and Wright and Semple's sedimentation-tube methods may be used (see pp. 185–8).

Choleraic dejecta.—From the characteristic *rice-water stool*, film preparations are carefully made and stained with carbol-fuchsin. If a large number of vibrios are seen, as in the case from which the specimen (fig. 104) was made in Professor Koch's laboratory, the diagnosis of cholera is practically at once established, as there is *no other disease in man, at present known, in which so many typical comma bacilli are found massed together in fæcal evacuations.*

But in all cases, cultures from flakes of the rice-water stool should be made, and tested exactly as in case of suspected water.

N. B. It is a good thing to keep the solidified gelatine plates *with cover-side down*, when incubated at 20°–22° C., for the first night, so that the moisture which soon collects inside the cover when kept in the natural position shall not run down on to the medium and blur the outline of the colonies. Subsequently the plates should be kept in the natural position, top-side up.

To sum up: a vibrio, suspected of being Koch's comma bacillus, must first be isolated in pure culture from colonies which appear to bear a resemblance to those of the organism in question. It must then give the cholera-red (indol) test, and be pathogenic when inoculated into the peritoneal cavity of a guinea-pig; and lastly, when mixed in proper proportions with the serum of an animal which has been immunised against Asiatic cholera, Pfeiffer's, or Durham-Gruber's test, must be given (see p. 200).

Unless the suspected vibrio gives all these tests, the proof that it is Koch's Comma bacillus is wanting.

Varieties of cholera bacilli.—It should here be mentioned that the bacilli obtained from different cholera epidemics vary so much in shape and size that it is now generally believed that there is a multiplicity of cholera bacilli (Cunningham). The *Massowah* cholera bacillus is one of the chief varieties. It is elongated, and very thin; but, apparently, a similarly shaped bacillus can be experimentally derived from the short comma-form found at Angers.

The Durham-Gruber reaction is only obtained when the organism (*e.g.* Massowah) is mixed with the diluted serum of an animal immunised against the specific organism in question (*e.g.* the Massowah bacillus). While Koch's comma bacillus has a single flagellum at one end only, the Massowah and some other similar varieties have four flagella, two at each end.

Results of inoculation.—To produce any effect with guinea-pigs, the gastric juice must be rendered slightly alkaline, or peristalsis must be delayed by morphia. Subcutaneous, or intravenous, injections, otherwise, also give negative results.

It has been shown by Metchnikoff that newly born rabbits (one to four days old) die, if cholera bacilli, especially when associated with other usually harmless organisms (torula, sarcina), are mixed with their food, with typical symptoms and *post-mortem* appearances.[1] The pathogenic effects of cholera bacilli injections are due to a soluble toxin, which has recently been isolated.

Of the numerous other organisms more or less resembling Koch's comma bacillus, the two vibrios described by Finkler and Prior, and by Metchnikoff, respectively, deserve careful consideration.

Vibrio, or Spirillum, of Finkler-Prior.—This organism, discovered by Prior and Finkler, was one of the earliest brought up in opposition to Koch's claim to have discovered the bacillus of Asiatic cholera. These observers stated that they had found a curved organism, in the dejecta from cases of Cholera Nostras, identical with Koch's comma. Subsequent investigation proved without doubt that the vibrio of Finkler and Prior is not the same as Koch's vibrio, nor has it since been found in a series of cases of Cholera Nostras. Both by the various reactions and inoculation experiments, it can readily be distinguished from the true comma bacillus.

Cultivations.—*Gelatine stab.*—After twenty-four hours' incubation at 22° C., while, in the case of the cholera organism, the growth along the track of the needle is hardly perceptible except as a fine line, in the case of vibrio Finkler-Prior (fig. 97, c) there is a distinct growth with liquefaction in the upper part of the track, and an air-bubble has already appeared, owing to the evaporation which has occurred from the liquefied gelatine. In forty-eight hours, this distinction is still more marked, the gelatine being rapidly liquefied in the upper part of the tube, the liquefaction extending downwards in the shape of a cone with its base upwards, the point of the cone being indicated by a flocculent opaque white growth. This point subsequently widens out (fig. 99), and may be bent to form, as it were, a foot-piece, which is set at an angle to the rest of the conical down-growth ; so that the appearance presented, by the third or fourth day, has been compared with that of a 'stocking-foot.' (This, however, is not shown in the figure.)

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[1] *Outlines of Bacteriology*, Thoinot and Masselin, edited by Symmers, p. 220.

Gelatine plates.—In from twenty to twenty-four hours, the colonies (fig. 100, c) have developed to about twice the size of those seen in the case of cholera organism, with considerable liquefaction of the gelatine. Further, the colonies, under a low power of the microscope, appear darker, more granular, and more distinctly circular, the somewhat lobulated appearance and crenated margin, noticed in the case of cholera, being, as a rule, less evident. After forty-eight hours, liquefaction is very marked, the white colonies floating in clear liquefied gelatine, which is limited by a sharp, circular outline, the whole colony being somewhat sunken in the surrounding gelatine, owing to the evaporation which has occurred after liquefaction.

The appearance presented by the plate colonies, when seen under the microscope, on the third day, serves to distinguish this vibrio from that of cholera (*cf.* fig. 102, A, and C) at the same stage, the central mass of the colony is very much more ragged, branches spreading very irregularly into the surrounding liquefying gelatine. The double contour effect produced in the case of cholera colonies is less marked, as a larger, clearer area results from the more rapid liquefaction which occurs in the case of the vibrio Finkler-Prior.

Agar-agar.—The growth consists of moist, greyish-white, or yellowish-white colonies, not to be distinguished from those of cholera.

Potato.—The growth, even at the temperature of the room, is abundant, and of a darker yellow colour than in the case of cholera.

No indol reaction can, as a rule, be obtained on the addition of nitrite of sodium and concentrated sulphuric acid. A slight reaction, however, may be given after three or four days, in the case of old stock cultures.

Inoculation experiments.—As in the case of cholera, negative results have followed injections subcutaneously, and intravenously, and feeding with the cultures. If, however, the acidity of the gastric juice be counteracted by a weak solution of sodium carbonate (*e.g.* for a guinea-pig of medium weight, 5 c.c. of a 5 p.c. solution thereof), especially if peristalsis of the alimentary canal is checked by a hypodermic injection of tincture of opium, death occurs in less than forty-eight hours, in about a third of the animals so treated.

Under the microscope, the organism appears (fig. 105) as a comma-shaped bacillus, in young cultures hardly distinguishable from those of cholera. It is, however, slightly thicker in the middle than at the extremities ; and eventually it becomes distinctly longer than Koch's vibrio. Two vibrios are frequently joined end to end, their concavities pointing in the same direction. They may point in opposite directions, or a third may be added so as to form a spirillum of considerable length. In coverslip preparations, the bacilli, interlaced with one another, may sometimes be seen much curved and arranged concentrically, forming a series of horseshoe-shaped filaments.

Impression preparations of colonies on gelatine plates (fig. 105) enable one to obtain healthy organisms when streak cultures, made on agar &c. in the ordinary way, may have produced only involution forms, degeneration occurring very readily in the case of all these vibrios, Koch's comma bacillus, V. Finkler-Prior and V. Metchnikovi. Further, the organisms in the case of vibrio Finkler-Prior do not appear to be so regularly arranged, like a school of fish swimming up stream, as in the typical colonies of cholera bacilli.

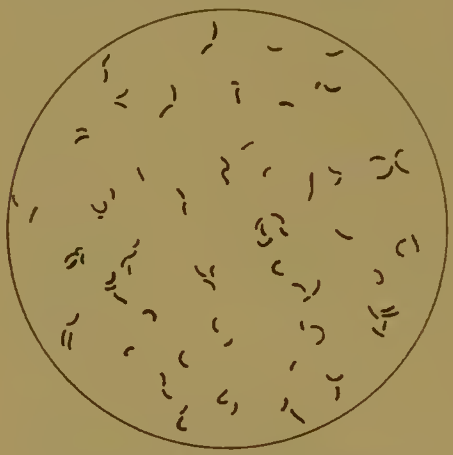

Fig. 105.—Vibrio Finkler-Prior × 530

Gelatine plate, three days old. Impression preparation.

In a hanging-drop, the motility is well marked and is associated with the presence of a single flagellum, which may be demonstrated by means of Van Ermenghem's, or other special methods of staining them.

Vibrio Metchnikovi.—This organism was discovered in large numbers in the intestinal contents of fowls, noticed to be dying of gastro-enteritis, in the Odessa markets. It perhaps more closely resembles Koch's comma bacillus than the vibrio Finkler-Prior. It can, however, be fairly easily distinguished from the organism producing cholera.

Cultivations.—*Gelatine stab.*—As will be seen on comparing the series of plates (figs. 97–99), the growth of the vibrio Metchnikovi appears more rapidly along the track of the needle, and at the same time, liquefies the gelatine at a far greater rate than is the case with the true cholera vibrio. Comparing the three organisms, the vibrio Finkler-Prior liquefies the most rapidly, Koch's comma bacillus the least rapidly, and the vibrio Metchnikovi is intermediate in this respect.

After four or five days, liquefaction proceeds so rapidly in the stab culture of the vibrio Metchnikovi as to destroy its characteristic appearance.

Gelatine plates.—The differences between the colonies of the cholera vibrio and those of the vibrios of Finkler-Prior and Metchnikoff respectively, are well demonstrated by contrasting the appearances seen in figs. 100–102. There appear to be, broadly speaking, two types of colonies in the case of V. Metchnikovi, one having crenated edges and a lobulated appearance, not unlike those of Koch's comma, the other type, after forty-eight hours, more closely resembling those of the V. Finkler-Prior, having a dark centre spreading out by ragged edges into the cup-shaped disc of liquefying gelatine. The periphery of such colonies can, by careful focussing, be frequently seen to be finely striated.

Agar-agar.—The growth appears rapidly, is raised and moist, and of a greyish-white, or yellowish-white tint, similar to that noticed in the two last-mentioned organisms.

Potato.—The growth is raised, moist and of a dirty brownish colour, not unlike that of the *B. coli*, but not spreading so rapidly as it over the surface of the medium. This contrasts markedly with the hardly visible growths in the case of the cholera vibrio, and the lighter tint seen in the case of V. Finkler-Prior.

Nutrient broth, and peptone-water.—Turbidity is marked after twenty-four hours. In the case of the broth, a thin scum is eventually produced. In the peptone-water (as well as in the broth, which contains peptone) the indol reaction can be obtained on the addition of five or six drops of concentrated sulphuric acid alone, without the use of nitrites, which are formed with the indol by the organism. The red colour which appears on doing the indol reaction is often much deeper in the case of V. Metchnikovi than in the case of Koch's comma bacillus.

Inoculation experiments.—Fowls and guinea-pigs, when injected subcutaneously with this organism, rapidly die from gastro-enteritis, with profuse diarrhœa. Death occurs in from twenty to twenty-four hours, apparently from an acute septicæmic condition. At the post-mortem examination, necrosis is seen locally at the seat of inoculation; and there is œdema of the tissues in the neighbourhood, where the vibrios swarm. Great congestion of the entire length of the alimentary canal is found, the intestinal contents consisting largely of blood-stained yellowish fluid. Pigeons, inoculated intramuscularly with this material, die in from eight to twenty hours, the muscle fibres being found necrosed. There are also marked swelling and œdema of the muscular tissue. Large numbers of vibrios are found in the inoculated muscles, the blood, and the viscera, as happens in the case of guinea-pigs (Abbott).

Under the microscope, the bacilli are seen to be comma-shaped organisms (fig. 106), which—especially in the early stages of their growth—are hardly to be distinguished from true cholera vibrios. They may be shorter; on the other hand, they may be much longer than the cholera bacilli, and they are generally thicker, both in the centre, and at the poles, than is the case with Koch's comma bacillus.

In a *hanging-drop*, the vibrios are seen to be motile, and a single flagellum can be demonstrated by the usual methods.

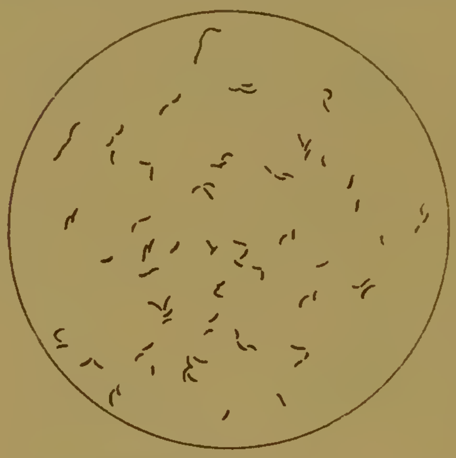

Fig. 106.—VIBRIO METCHNIKOVI. × 530

Gelatine plate, three days old. Impression preparation.

A large number of vibrios, or spirilla forms—such as that of Deneke, found in cheese (*Spirillum tyrogenum*), and *Miller's spirillum*, found in carious teeth—more or less resembling the organism of cholera, have been isolated, and of late years much attention has been paid to this subject by Sanarelli; but the two which have been referred to in detail, V. Finkler-Prior and V. Metchnikovi, are perhaps the most important for students to be familiar with. What has been said of them will serve to indicate the mode by which other pseudo-cholera bacilli may be distinguished from the true organism.

LESSON XIX

ACTINOMYCOSIS—MADURA DISEASE, OR MYCETOMA—MOUSE SEPTICÆMIA—INFLUENZA—BUBONIC PLAGUE—YELLOW-FEVER

A. **Actinomyces**
B. **Streptothrix maduræ**
C. **Bacillus of mouse septicæmia**
D. **Influenza bacillus**
E. **Bacillus of bubonic plague**

A. Actinomyces

I. *Cultivations* on (i) Glycerine agar.
(ii) Potato.

II. *Stain coverslips* α. *Cultures* (i) with carbol-fuchsin.
(ii) by Gram's method.
β. *Pus*, from a recent case of the disease; use the Gram-Eosin method.
γ. Paraffin sections of actinomycosis in the tissues—*e.g.* cow's tongue.

The tissue may be stained in bulk with carmine, and the organism then counterstained by the Gram-Weigert method; or, if not stained in bulk, the Gram-Eosin, or Eosin-Gram-Weigert, or Carmine-Gram-Weigert methods may be used.

B. Streptothrix maduræ

Cultivations, *film preparations*, and *sections of diseased tissue* may be treated as in the case of A., if the material is available.

C. Bacillus of mouse septicæmia

I. *Cultivations*: (i) Gelatine stab.
(ii) Gelatine streak.
(iii) Glycerine agar streak.

II. *Stain film preparations of cultures* (i) with carbol-fuchsin.
(ii) by Gram's method.

D. Influenza bacillus

I. *Cultivation.* Make an emulsion of sputum with nutrient broth, from a case of influenza. (Refer to p. 218.) Inoculate a previously prepared, glycerine agar plate streaked with fresh blood, and shown to be sterile after incubation at 37° C.[1]

II. *Stain films* of influenza sputum, or of culture, } with carbol-fuchsin.

E. Plague bacillus

I. *Stain film preparations of a sterilised culture*:
(i) With anilin-gentian violet, or carbol-fuchsin;
(ii) By Gram's method (*the organism is decolorised*).

II. *Stain sections of organs* from an animal affected by the disease.

The method recommended by Hewlett for glanders bacilli in tissues (p. 154) may be adopted.

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[1] For other methods of making blood glycerine-agar plates, see pp. 125–126.

Actinomycosis is a disease affecting cattle, especially cows. Among other animals commonly affected may be mentioned the pig and the horse ; and, comparatively recently, careful clinical observations have shown that the disease is not at all uncommon in man, especially among those whose occupation brings them much into contact with straw—farmers, grooms, &c. But it also occurs amongst other people not obviously so associated with straw.

In cows, the tongue is frequently affected ; and when cut, a grating sensation is perceived, owing to the induration produced by the disease, which is appropriately known as 'wooden tongue.' When occurring in the jaw—another common site of the lesion—it has been termed 'osteo-sarcoma,' or 'bony sarcoma ;' when seen in the roof of the mouth it has been called a malignant tumour of the palate. But the disease is not confined to the jaws and tongue : it may also affect the pharynx, or the skin and subcutaneous tissues of any part of the body, as also the region of the appendix.

In the pig, similar regions may be affected, but the disease in this animal appears to be associated most frequently with abscesses forming running sores in the milk gland (Sims Woodhead). The lungs and the pleura, the bones of the vertebral column, and elsewhere, especially if containing expanded air cells, are also sometimes affected in this animal, as is very frequently the case also in man.

In horses, the disease is rarer than in cows, and produces the condition known as 'scirrhus cord.' Rarely the tongue is affected. The gravity of this affection makes an early recognition of it, in animals and man, all important.

The disease assumes two main types, of which the 'wooden tongue' in cows and the 'cold abscess' condition commonly seen in man are examples. If the diseased tongue of a cow be seen in section, it is observed to be studded with minute, yellowish-white points, or nodules, situated in the sub-epithelial, or muscular layers. From a pathological point of view, apart from the fungus to be described, the indurated mass produced is essentially a granuloma, consisting of a mass of round cells, enclosed in a fibrous, more or less well defined, capsule. The nodules, in cows, are said never to break down into pus. In man, on the contrary, the disease most often produces abscesses of the superficial or deep parts, of which the parasitic origin may be readily overlooked, and thus the pus should, in all suspicious cases, be carefully examined. In the human subject, the disease may affect the jaw and produce chronic sinuses, leading down to the bone, from which pus, containing characteristic yellow bodies, may escape in the discharge.

Sinuses may be met with in connection with the ribs and other bones, and where they are in the region of the thorax, they not uncommonly indicate the existence of mischief in the lungs, and neighbouring organs. Thus, in a case at University College Hospital, in 1896, the disease affected almost the whole of the right lung and extended through the diaphragm into the liver, which in section showed a very honeycombed appearance throughout.[1] It may also extend from the thorax into the anterior abdominal wall, to form a fluctuating, rounded swelling, very easily mistaken for a 'cold abscess.'

The skin immediately surrounding such sinuses is of a dull leaden, or purplish tint; and from cloaca-like openings with protuberant lips, composed of granulation tissue, pus escapes containing characteristic, opaque, lemon-yellow bodies. These are often not larger than a small pin's head; they are circular, or slightly oval. When placed on a slide and pressed flat with a coverslip, they may be examined with the microscope unstained. The periphery of the mass is seen to be made up of a more or less radiating fringe of club-like processes. Hence the name 'actino-myces,' *lit.* 'ray-fungus' (*cf.* fig. 111). These clubs appear to be arranged layer upon layer, as seen by repeatedly altering the focus, while examining this flattened-out mass; and in the centre, the elongated clubs appear to be replaced by smaller rounded cells, which are really pointed tips of other clubs seen in optical section. In many parts of the field, single clubs are seen, freely scattered about.

Cultivations.—The two best media for the growth of the actinomycosis fungus are glycerine-agar and potato.

Glycerine-agar streak.—After a few days at 37 C., small, discrete, glistening white colonies appear, and by their multiplication they become heaped up, but do not really coalesce (fig. 107, A). In the course of a week or so, the tint becomes yellowish or light brown, and subsequently the whole culture becomes of a dirty greenish-brown colour. Some cultures remain with the colonies in this discrete condition for many months. Very frequently, however, the condition just mentioned is replaced by a lichen-like growth of a yellowish, greenish-white, or ash-grey tint (fig. 107, C).

Another type of culture commonly seen on agar (fig. 107, B) is almost circular in outline and considerably raised above the surface, so as to produce a very irregular cone, or limpet-shell, appearance. It is of greenish-brown tint.

Potato.—The growth is of a yellowish or greenish-brown colour, which turns to an ash-grey tint as the size of the colony increases. The growth soon becomes exuberant, so that it forms a mass almost as big as, if not bigger than, the half cylinder of potato

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[1] An almost identical condition is figured on p. 417 of Crookshank's *Bacteriology*.

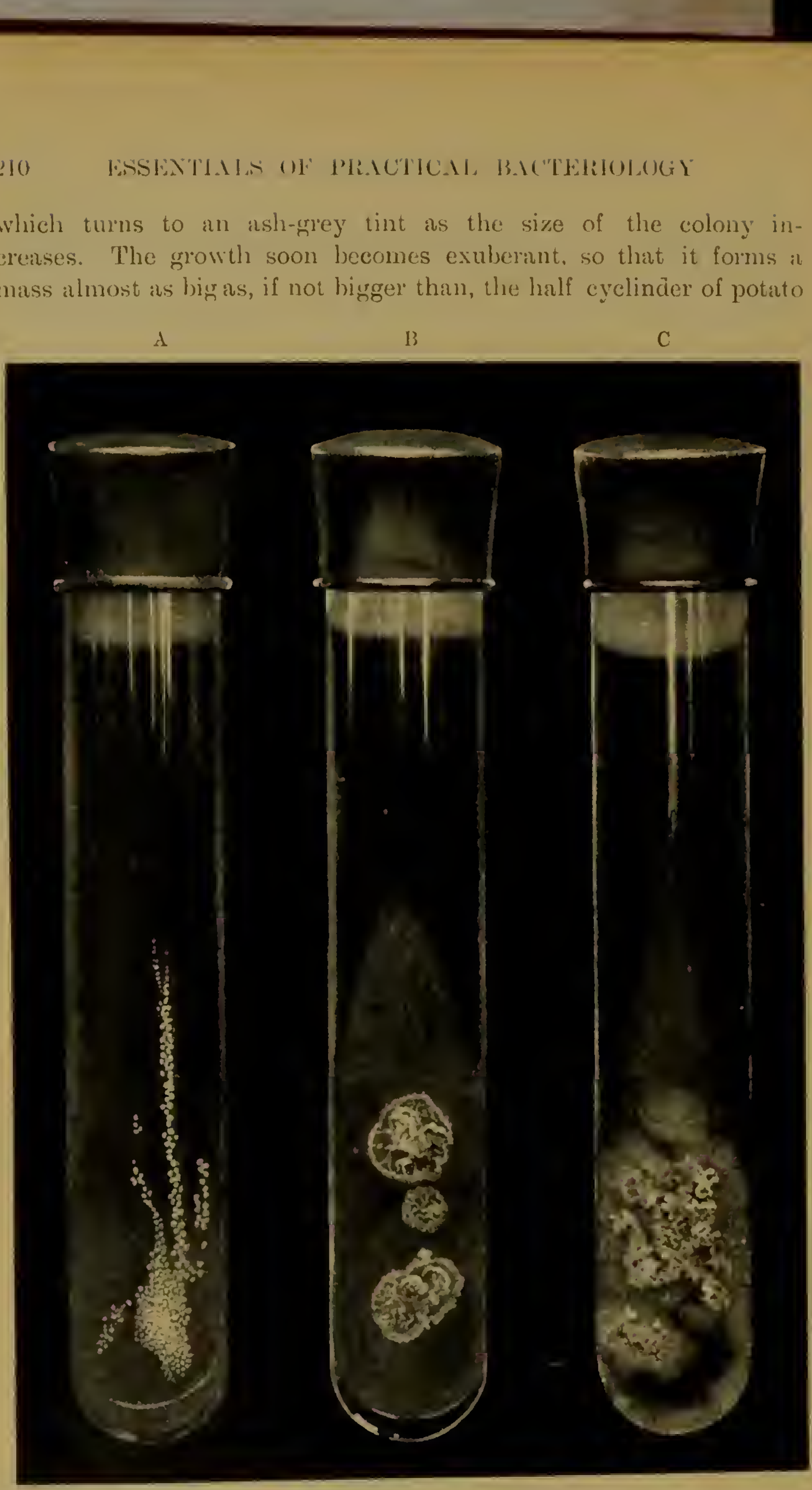

FIG. 107.—ACTINOMYCOSIS

Glycerine-agar cultures. A, discrete rounded colonies, after about ten days' incubation at 37° C. B, limpet-shaped colonies, three and a half months old. C, lichen-like appearance frequently seen; the growth is three and a half months old.

on which it has been inoculated. The potato, itself, acquires a darker tint. It is not always easy, however, to grow the organism on potato, doubtless owing to the varying conditions of acidity, &c., in different kinds of potato.

Bread paste has been recommended as a favourable medium. It is prepared by drying bread, freed from its crust, at a high temperature; it is then broken up into a fine powder in a mortar, or it is passed through a fine sieve. After placing it in a sterile flask, it is made up into a paste by mixing with it water, in the proportion of one of water to four of powder. The flask with its contents is then sterilised in the usual way for three successive days in a steamer, for twenty to thirty minutes.

Gelatine has been used, the growth consisting of 'small, discoid, whitish colonies, that soon liquefy the medium and subside into the clear fluid, as spheroidal grains which retain the same white colour' (Shattock).[1] The *Streptothrix Maduræ* does not liquefy gelatine.

Blood-serum.—A growth occurs, thus again differing, be it noted, from the organism of Madura foot, otherwise so similar, which will not grow on this medium.

Under the microscope, the organism, best stained by Gram's method, is seen, in young cultures, to consist of rounded, cocci-like bodies, in the midst of which bacilli, varying in length from short rods to long threads, appear. With the increase in age of the culture, these threads branch out, and frequently themselves appear to stain irregularly, so that they look as if made up of a line of minute cocci (fig. 108). The free extremities of the branches frequently appear thickened and club-like, especially in old cultures, and in pus (fig. 109). This condition of clubbed extremities tends to confirm Böstrom's view that the clubs which form the extremities of the radiating processes, making up the typical 'ray-fungus' in sections of the diseased tissues, are really nothing but degenerations in the sheath of the fungus, and that, therefore, they are not really indications of spore formation.

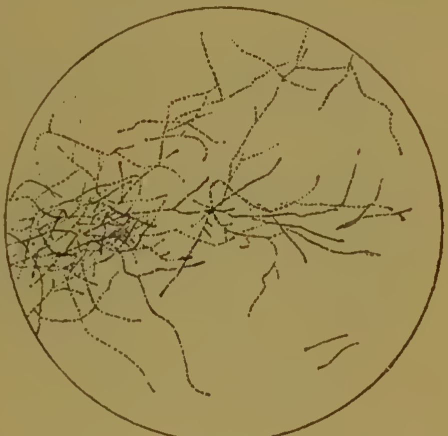

FIG. 108.—ACTINOMYCES. × 530

Pure culture on glycerine agar, twenty-five days old. Note the branching of the filaments, many of which are clubbed at their extremities. The very granular appearance of the filaments is commonly seen in old cultures.

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[1] *Trans. Path. Soc.* 1896, p. 381.

Böstrom, quoted by Sims Woodhead,[1] has further shown that, from the club-like extremities of the fungus no growth on ox-blood serum and agar-agar results, though the filaments forming the mycelium proper in the central part of the ray-fungus can be grown on this mixture. The growth, on the medium referred to, first appears as fine granules, which subsequently develop into small, yellowish-red nodules, from which fine branching processes spread out. Later, by about eight days, the yellowish masses have run together and are covered with a delicate fluffy white layer.

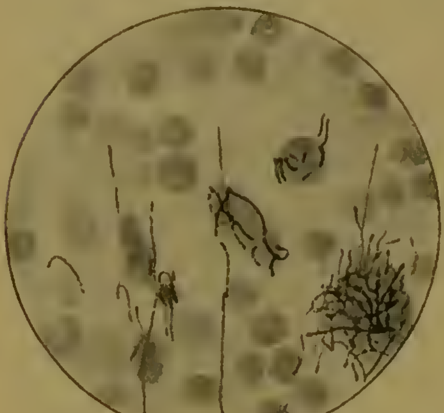
FIG. 109.—PUS FROM A CASE OF ACTINOMYCOSIS. × 530

The mycelial filaments are readily demonstrated by staining the pus by the Gram-Eosin method.

Pus.—Besides the opaque yellowish particles, already described as occurring in pus from cases of actinomycosis, felted mycelial filaments may often be seen microscopically, even if there are no opacities evident to the eye. The filaments resemble, in general appearance, a pure culture (*cf.* figs. 108 and 109). Dense masses of mycelium, with branched, and sometimes slightly clubbed, extremities, can be easily rendered visible on staining the pus by the Gram-Eosin method.

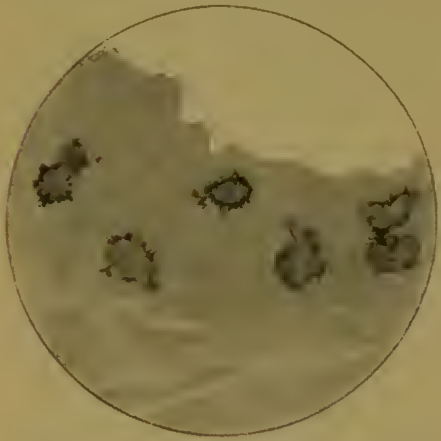
FIG. 110.—ACTINOMYCOSIS AFFECTING THE LIVER OF A COW. × 50

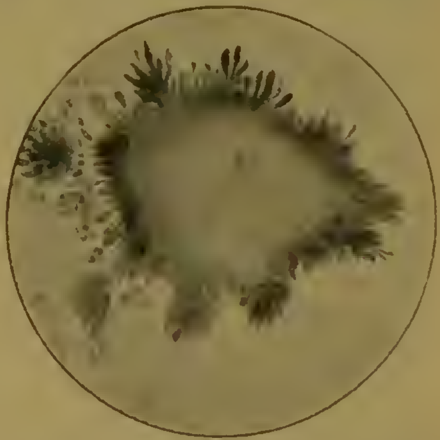
FIG. 111.—ACTINOMYCOSIS AFFECTING THE LIVER OF A COW—ONE OF THE FUNGI SHOWN IN FIG. 110. × 530

Sections of diseased tissues.—In the indurated masses, such as are found in the tongue of the cow, or in the liver of the same animal (see figs. 110, 111), a characteristic ray-like appearance is presented. The mass is irregularly circular, the centre is often obscure; but on careful fixing and staining by special methods, it may sometimes be seen to be made up of a fine mycelium. The periphery consists of a series of elongated processes set side by side like a palisade, the outer extremity of each process forming an elongated oval, slightly pointed at its tip, and being thus somewhat club-shaped. It should be stated that in man the clubs are frequently absent in actinomycosis, and also—in exceptional cases—in cows, the organism in the two cases having been shown, however, to be identical, the forms being interchangeable. This is an important point, and much emphasised by Shattock in a criticism of a valuable paper on the *Streptothrix Maduræ*, by Vincent,[1] who stated therein that the micro-organism of Madura foot, when examined in the natural condition, has no clubs, and so differs from actinomyces. As a matter of fact, they are not invariably absent from Madura streptothrix, being figured by Kanthack[2] and Hewlett.[3]

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[1] Treves's *System of Surgery*, vol. i. p. 50.

Around the fungus there is a collection of young cells, enclosed in a fibrous capsule, more or less defined. The central part of such nodule is often soft, and minute points of caseation are not uncommon.

In the human subject, although the ray-fungus can very readily be shown in the pus, in the tissues, on the contrary—*e.g.* from the liver—when broken down into a honeycomb-like mass, as described above, the greatest difficulty may be experienced in demonstrating the fungus *in situ*.

Results of inoculation.—Intraperitoneal inoculation of cultures in the case of the rabbit, and injection subcutaneously in the case of calves, have succeeded. Frequently, however, a negative result follows.

Madura Foot, or Mycetoma

Streptothrix Maduræ.—*Madura disease*, *Madura foot*, or *Mycetoma*, was formerly thought to be confined to India, but is now known to exist in Italy, Morocco, Algiers, and America. The disease, according to Vincent,[1] begins as a diffuse swelling of the integuments of the foot, rarely of the hand. Small tumours are formed, and the pus escaping from fistulous openings contains yellowish rounded bodies, about the size of a grain of semolina. They are insoluble in potash and acetic acid, and consist of streptothrix filaments, forming a fine densely interlacing mycelium—the *Streptothrix Maduræ*, the peripheral filaments of which give off lateral offshoots, as in the case of actinomyces.

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[1] *Ann. de l'Instit. Pasteur*, 1894.

[2] *Journ. Pathol.* 1892.

[3] *Trans. Path. Soc. Lond.* 1893.

[1] *Loc. cit.*, quoted by Seligmann (*Trans. Path. Soc. Lond.* 1896), whose paper I have followed in the present account.

Besides this '*pale or ochroid variety*,' which has been compared with fish roe, there is another '*black variety*,' consisting of a dark, granular substance, which has been likened to coarse gunpowder (Makins).[1]

A great difference of opinion has arisen as to the nature of these two varieties, Kanthack believing, at one time, that both were forms of actinomyces, the relation between the two being indicated by intermediate, degenerate, yellowish-black bodies. Boyce and Surveyor seem, however, to have established the fact that the two varieties are quite distinct, 'the yellow masses being for the most part composed of caseating material, in which the fungus, possessing some of the characteristics of actinomyces, is to be found, while the black masses are somewhat more highly organised and have larger branching filaments.' Both fungi are believed to be pathogenic, but are considered to be distinct from one another. The extremely close resemblance between the organisms of Madura foot and actinomycosis is very evident, and, according to Shattock, their primary source may prove eventually to be identical.

Inoculation experiments on rabbits, guinea-pigs, cats, and mice have uniformly failed (Vincent).

Cultivations.—*Nutrient broth* may be used, but the growth is sparse, slowly forming flocculent zooglœa masses, as also in the case of hay infusion.

Hay-, or potato-, infusion kept at 37° C. appears[2] to be the best medium. Greyish flocculent zooglœa masses, sticking to the glass, appear about the fourth or fifth day. In three weeks they are as large as a pea, and may have a brownish centre. In a month or two, colonies near the centre become pink or red; a mass of whitish spores may form a sort of scum on the free surface of the liquid. In the case of actinomyces, it may be stated, no growth occurs.

Gelatine, to which have been added glucose and glycerine, gives rise to abundant, convex, yellow colonies, with glazed surface. They may then become dark red, the white centre becomes depressed, this umbilication of the hemispherical colonies contrasting with the wrinkled surface of actinomyces. (Shattock, in criticising this last statement of Vincent, says that the umbilication is not confined to the Streptothrix Maduræ, but is often seen in the case of actinomyces grown on grape sugar.) There is no liquefaction of the gelatine, as occurs in the case of actinomyces.

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[1] Treves's *System of Surgery*, vol. i. p. 338.

[2] Seligmann, *loc. cit.*

Glycerine agar.—Growth occurs slowly at 37° C., and is white or unpigmented.

Potato.—The growth is red, dark red, or maroon colour, the potato itself not darkening in colour. In actinomyces, on the contrary, the growth is dark ash-grey, or yellowish-white in colour, and very much raised, the potato itself becoming darker in tint.

Under the microscope, streptothrix filaments, closely resembling those of actinomyces, are seen; and, though clubs were absent in Vincent's cases, they have been seen by other observers, Kanthack,[1] Hewlett,[2] &c. Bent filaments, probably involution forms, with irregular thickenings, and lacunæ in the protoplasm, are mentioned by Vincent.

The Bacillus of Mouse Septicæmia

Mouse septicæmia.—This condition often results from the inoculation of house mice with a small quantity of putrescent material. The animal becomes obviously ill, and refuses to move about. It remains quite passive, and a gummy discharge, as the result of conjunctivitis, may seal up the eyelids (Crookshank). Death occurs in two or three days. Rabbits and swine are also very susceptible. Field mice are insusceptible. At the point of injection there is, in the case of mice, often an œdematous swelling, the spleen is enlarged, and in the tissues, especially in the capillaries, a minute organism 1 μ in length, called the *Bacillus murisepticus*, discovered by Koch, is seen in large numbers.

It is believed to be identical with the organism of Swine Erysipelas, which, in turn, some have suggested as probably being identical with the organism associated with Membranous Colitis in man.

Inoculation with a pure culture, in the case of house mice, produces similar effects to those just described. In sections of the lung, spleen, &c., the capillaries are seen crowded with bacilli, which are found in the leucocytes in such numbers as to give the white corpuscles a very characteristic, and highly granular appearance. Guinea-pigs and rabbits inoculated subcutaneously in the same way show only a slight local reaction (œdema, &c.). When the culture is rubbed into the conjunctiva, however, conjunctivitis with characteristic gummy discharge sealing the eyelids, results, according to Crookshank.

Cultivation.—*Gelatine stab.*—A very delicate, brush-like, growth appears, spreading out in all directions from the track of the needle

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[1] *Journ. of Path.* October 1892

[2] *Trans. Path. Soc. Lond.* 1893.

(fig. 112). No liquefaction of the gelatine occurs, unless it is very alkaline, when it sometimes results (Woodhead).

Gelatine streak.—The growth consists of minute, slightly raised colonies, many of them being hardly visible at first, except when by transmitted light. They are seen then more perceptible, but never become very distinct.

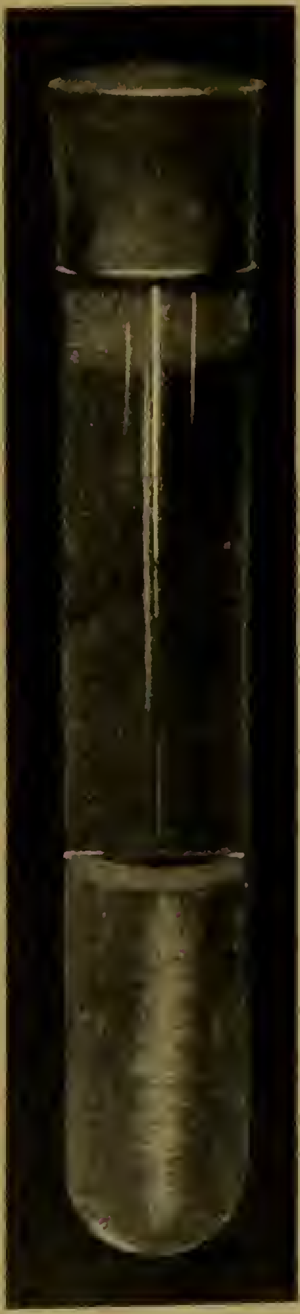

FIG. 112.—BACILLUS MURISEPTICUS (MOUSE SEPTICÆMIA)

Gelatine-stab culture, twelve days old.

Agar-agar streak.—The growth is similar to that seen on gelatine, the surface being powdered over with minute, hardly perceptible colonies, often no larger than a pin-point.

Under the microscope, the bacilli of mouse septicæmia (said to be the shortest of all *bacilli* except that of influenza) are seen to be arranged singly, frequently in pairs, and sometimes in threads, in which, however, the divisions are usually readily made out on focussing. The individual bacilli are minute rods with rounded ends, 1 μ long and $\frac{1}{10}$ to $\frac{1}{5}$ of their length in thickness. Spore formation has been described.

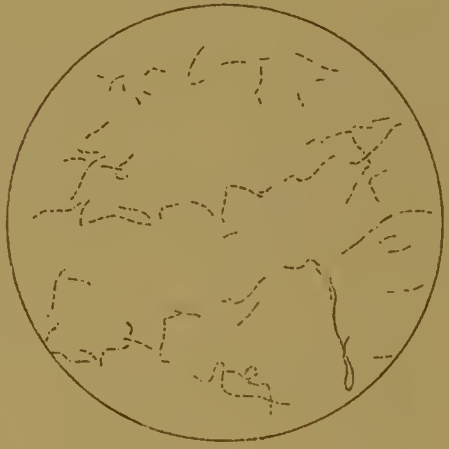

FIG. 113.—BACILLUS MURISEPTICUS (MOUSE SEPTICÆMIA). × 530

Pure culture.

For further information, the larger

text-books should be consulted, especially as to the large number of other forms of septicæmia affecting animals.

Influenza

Influenza, or 'La Grippe,' to use the French name, has occurred from time to time in widespread epidemics. It appeared many times between the sixteenth and eighteenth centuries in this country, and frequently between 1800 and 1850. After a long interval it re-appeared in 1889, since which time there have been several distinct outbreaks. The 1890 epidemic is supposed to have originated in Bokhara, in Turkestan, whence it spread over Russia, Germany, and France to England. Its incubating period is said to be a few hours only, so that sailors, perfectly healthy at the time of landing at an infected port, are reported to have been attacked in large numbers by the disease in an incredibly short space of time.

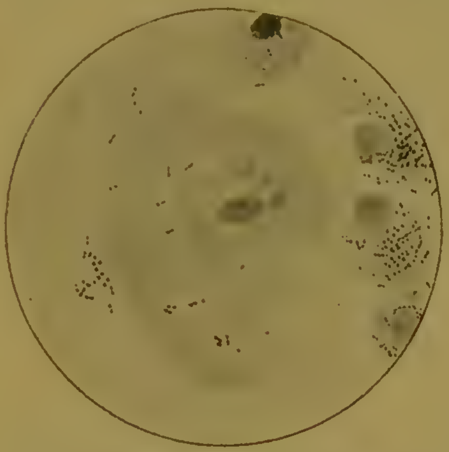

FIG. 114.—INFLUENZA SPUTUM. × 530

The halo-like capsule around the numerous groups of two bacilli in the interior of the large epithelioid cell is well seen.

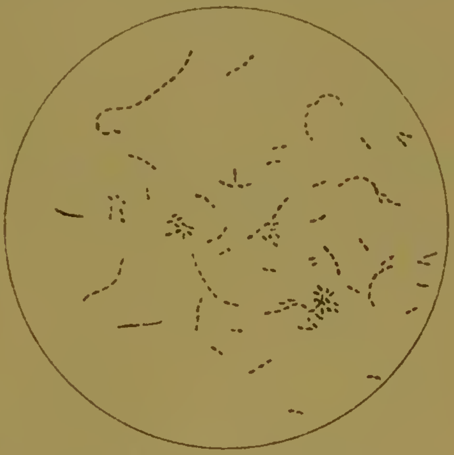

FIG. 115.—INFLUENZA BACILLUS. × 530

Pure culture on glycerine-agar.

As to the organism itself, the bacillus of influenza was isolated by Pfeiffer from the purulent bronchitic sputum; and Canon claims to have detected it in the blood. It has also been found after death in the peribronchial tissue. The greenish-yellow, muco-purulent, bronchial secretion from a case of influenza is crowded throughout with the specific organism (fig. 114), and the appearances to be described render the diagnosis very easy, there being no other disease known in which such crowds of minute bacilli occur in the sputum. In cases of phthisis supervening upon an acute attack of influenza, or in other very chronic affections which have arisen in this way, the bacillus may be detected for many months.

Cultivations.—*Glycerine-agar plates* streaked *with fresh blood*, in the usual way (p. 125), should be employed. An emulsion of the sputum is made in nutrient broth, and with it the surface of the blood-agar plate is inoculated.

Hewlett ('Manual of Bacteriology,' p. 281) recommends washing the sputum in several test-tubes of sterile normal saline solution, the patient having gargled his throat with hot water before expectorating.

Numerous colonies appear in twenty-four hours or so, and consist of small translucent points like dewdrops, which can hardly be seen without the help of a lens. According to Kitasato, these colonies never become confluent (Wurtz).

Canon states that he has, from a series of cases of influenza, cultivated the bacillus by allowing the blood to drip from a finger prick into a petri-dish containing glycerine-agar broth. Subsequent investigators have been less fortunate, and some are inclined to doubt the correctness of this observation.

Under the microscope, the organism, as seen in pure culture (fig. 115), consists of a very fine, short rod, with rounded ends. Though of about the same thickness as *B. murisepticus*, it is only half its length, *i.e.* 0·5 μ. The bacilli are frequently in chains, and but for their minute size might sometimes be mistaken for pneumococci. The organism of influenza is, in fact, one of the shortest of all known *bacilli*.

Carbol-fuchsin is an excellent stain. The organism is decolorised by Gram's method. For the demonstration of the bacillus in the blood, Canon recommends fixing the films with absolute alcohol for five minutes, then staining in the following mixture (which closely resembles Chenzinsky's mixture) :—

Concentrated aqueous solution of methylene-blue, 40 parts : eosin, $\frac{1}{4}$ per cent. solution in 70 per cent. alcohol, 20 parts ; distilled water, 40 parts.

The cover glasses are floated in this mixture (placed in a covered watch-glass) from three to six hours at 37° C. ; they are then washed in water, dried, and mounted in balsam. *The red corpuscles are stained pink, and the leucocytes with the bacilli in them, blue.*

Bubonic Plague, and the Bacillus Pestis

The plague. The most striking changes in the body of a person suffering from Oriental, or bubonic plague are seen in the lymphatic glands which are greatly swollen, and of doughy consistence. Section shows them to be intensely congested, blood-stained serum escaping freely from the cut surface. Suppuration may ensue, early or late, but may be absent. The glands of the groin, and of the axilla, are generally the first to be involved. The lymphatic glands, the spleen, the liver (fig. 116, B), the kidneys, and other organs, become crowded with the characteristic bacilli; in addition, there is well-marked cloudy swelling of the cells themselves.

Shortly before death the blood also becomes crowded with bacilli, and from it pure cultures can readily be made (fig. 116, A).

The plague bacillus (B. pestis) was discovered in 1894, independently, by Kitasato and Yersin in the blood and inflamed lymphatic glands.

Rats and mice are very susceptible to the disease; in fact, the

Note.—It should have been stated in the text that fig. 116 has been drawn from specimens kindly lent to me by my friend Dr. Christopher Childs, of the Hygiene Department, University College, London.

CURTIS'S BACTERIOLOGY.

To face p. 218 *in centre.*

media, an alkaline solution of peptone, 2 per cent., with 1 to 2 per cent. of gelatine being perhaps the best (Crookshank).

Broth.—The growth slowly appears after two or three days, as a somewhat granular, white deposit, at the bottom and sides of the tube, the supernatant fluid soon becoming clear. If fat be present, as pointed out by Haffkine, the growth hangs down from the droplet of fat into the clear broth, in the form of stalactites.

FIG. 116.—BUBONIC PLAGUE. × 530

A, film-preparation of *B. pestis*: glycerine-agar culture, made from the blood of an infected animal: some of the plague bacilli are seen to be uniformly stained. B, section of pig's liver, containing numerous plague bacilli.

Gelatine stab.—There is a white growth along the track of the needle, consisting of minute pin-point colonies, and the surface of the medium is seen to be covered by a thin layer of growth. No liquefaction occurs.

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[1] *Brit. Med. Journal*, p. 1588, vol. ii. 1899.

Glycerine agar streaks.—The growth is not very characteristic, the colonies being, for the most part, confluent; where discrete they form more or less circular, translucent discs, somewhat resembling staphylococci colonies, and when examined under a low power of the microscope, they are seen to have a somewhat wavy outline.

The most favourable temperature for incubation is 33° C., or from 33° to 37° C. The more virulent the bacillus, the more scanty the growth (Symmers).

Under the microscope, the *bacillus pestis* (fig. 116, A) appears as a non-motile, very minute, ovoid coccus, or cocco-bacillus; and in older cultures it appears as a short, fat rod, with rounded ends. It stains readily by simple anilin dyes, but is decolorised when treated by Gram's method. It is characterised by well-marked bi-polar staining, although some of the bacilli may be seen stained uniformly, in film preparations made from cultures, in which, also, the organisms frequently form chains of varied length (*streptobacilli*); whereas in the tissues they are apt to lie singly, although, as in the illustration (fig. 116, B), streptobacilli may also be seen. The bi-polar staining, seen in cultures, is also found in the bacilli present in the tissues, but in these is rather less obvious. They may sometimes be seen to be enclosed in a halo-like capsule, in parts of the tissue. No spore formation has been observed.

Results of inoculation.—Subcutaneous injection of the cultures, from the blood, or from other diseased tissues, produces the same changes as have already been described for man. At the site of inoculation there is well-marked œdema; the lymphatic glands then become greatly inflamed, and the whole of the body becomes involved. There is intense congestion of the viscera, leading often to acute hæmorrhage. The spleen is enlarged. According to Symmers, *in rats* the liver looks as if studded with miliary tubercles, the spleen being normal; *in the guinea-pig*, however, the liver appears normal, whilst the spleen appears to be affected by miliary tuberculosis.

Protective inoculation, by means of agar cultures—killed by heating to 58°-60° C., for half an hour—has been obtained in the case of horses and other animals; and the serum from such immunised animals has a certain amount of protective power.

Haffkine employs a combination of the plague bacilli with their extra-cellular toxins, a specially prepared culture being incubated for five or six weeks. This is then sterilised by heating to a temperature of from 65° to 70° C. A second inoculation is very desirable.

The treatment by Haffkine's method is still *sub judice*, but the following statistics, given by Hankin,[1] may be quoted. At Hubli, in India, during the week ending August 26, 1898, the plague was so virulent that among the uninoculated one man in nine was attacked; yet, at the same time, among those who had been inoculated twice, only one person in 755 was attacked.

Later in the year, when only about 150 uninoculated persons remained at Hubli, 106 of these were attacked, while among the twice inoculated (over 31,000 persons) only one in 1,320 was attacked.

One attack of the disease is said to increase the power of resistance against a second. There appears to be comparatively little risk of contagion, in the case of English doctors and nurses in attendance on cases of the disease, if the ordinary precautions are adopted.

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[1] Recent Reports on Plague in India. *Brit. Med. Journ.* p. 724, vol. ii. 1899. This is a review of Hankin's pamphlet entitled *The Bubonic Plague*, 1899.

LESSON XX

ANAEROBES

TETANUS—MALIGNANT ŒDEMA—QUARTER EVIL

A. **Bacillus of Tetanus**
B. **Bacillus of Malignant Œdema**
C. **Bacillus of Quarter Evil**

Of A, B, and C

I. *Cultivations*[1] *in*
    (i) Glucose gelatine stab.
    (ii) 1 p.c. Glucose agar-agar: (α) stab;
    (iii) ,, ,, ,, (β) 'shake.'
    (iv) 0·5 p.c. Formate-of-soda-gelatine, or -agar.
II. *Stain coverslips of cultures*, with carbol-fuchsin.
III. *Stain for spores*, by Möller's method (p. 47).

Anaërobes

The three **obligate anaërobes** (p. 36) of chief importance are the bacilli of tetanus, malignant œdema, and quarter evil. They are distinguished from the aërobes, hitherto studied, by their inability to grow in the presence of oxygen, so that special means have to be taken to ensure the absence thereof.

The general methods now usually employed for growing anaërobes may, therefore, be conveniently mentioned here.

Buchner's method, which is very simple (fig. 117), consists in inoculating a small tube of suitable medium with the anaërobe in question, and inserting it, plugged with wool, into a larger test tube, containing some substance which, by its great avidity for oxygen, soon exhausts the supply out of both inner and outer tube, after the larger one has been plugged with a rubber cap. The substance usually employed is a solution of pyrogallic acid in the proportion of one gramme of the acid to 10 c.c. of decinormal solution of caustic potash,[1] the mixture of the two being made immediately before use.

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[1] Other methods of cultivating anaërobes, mentioned in the text, should be demonstrated to the class.

This principle may be imitated by the use of cultures using up a large quantity of oxygen, the culture of anaërobe in the inner tube being surrounded by a broth culture of *B. subtilis*, or other similar organism. The anaërobe is thus allowed to develop in an atmosphere deprived of oxygen. This is indeed the original, but less convenient, method of Buchner.

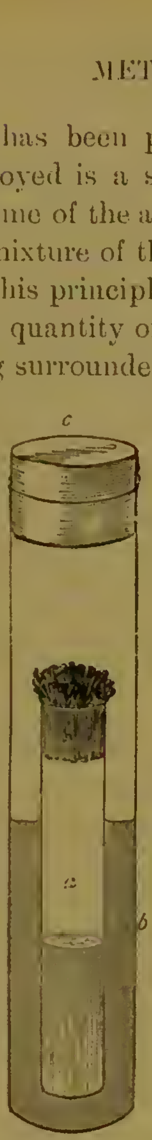

FIG. 117.—BUCHNER'S METHOD OF ANAËROBIC CULTURE

a, small test-tube culture; *b*, larger test-tube, containing pyrogallate of potash solution; *c*, indiarubber stopper. (From Schenk's 'Bacteriology.')

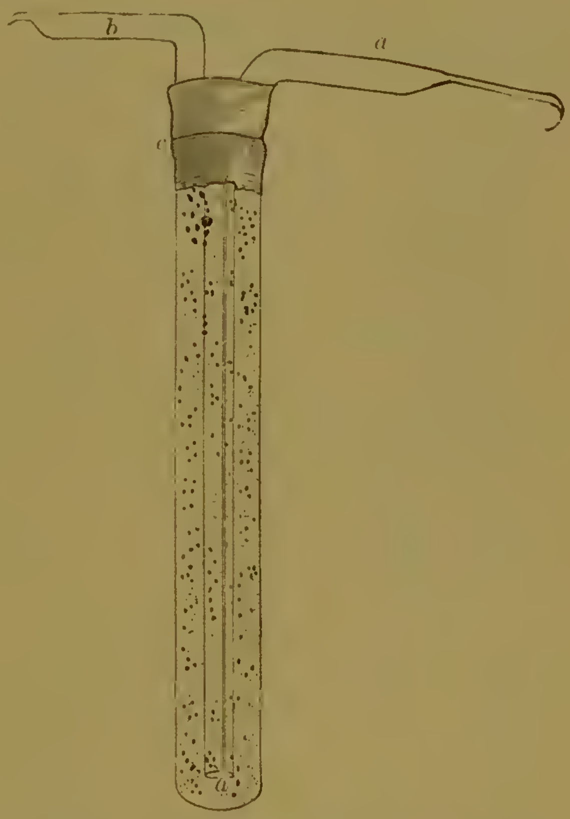

FIG. 118.—FRÄNKEL'S ANAËROBIC TUBE-CULTURE

a, glass tube through which hydrogen, or other gas, is passed; *b*, exit tube for gas; *c*, rubber stopper coated with paraffin. (From Schenk's 'Bacteriology.')

Fränkel's method.—A large Esmarch roll-tube (p. 21), containing a small quantity of liquefied gelatine or agar-agar, alone, or mixed with 1 p.c. glucose, is inoculated with the anaërobe.

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[1] The decinormal solution of caustic potash contains 5·6 grammes per litre of water. The decinormal solution of caustic soda, which may be used instead, contains 3·99 grammes to the litre of water.

The wool plug is rapidly replaced by a sterilised [1] rubber stopper fitted with two pieces of tubing, one of which passes to the bottom of the Esmarch tube, the other only just passing through the stopper (fig. 118). These pieces of tubing, after emerging through the outer surface, are bent at right angles to the stopper and are then constricted, so that they can subsequently be readily fused. By means of a Kipp's apparatus (fig. 119), hydrogen is generated and allowed to pass through a flask containing water, which absorbs any sulphuretted hydrogen present. On escaping from this flask the hydrogen passes through a second one, containing a mixture of pyrogallic acid and potash, in the proportion of 1 gramme of the acid to 10 c.c. of a decinormal solution of caustic potash. This deprives the gas passing through it of any carbon-dioxide, or oxygen, present. The hydrogen, thus purified, is then allowed to bubble in a continuous stream through the already inoculated gelatine, or agar, which is kept liquefied by placing the culture tube in water kept at 37° C., in the case of gelatine; or at from 40 to 42° C., in the case of agar.

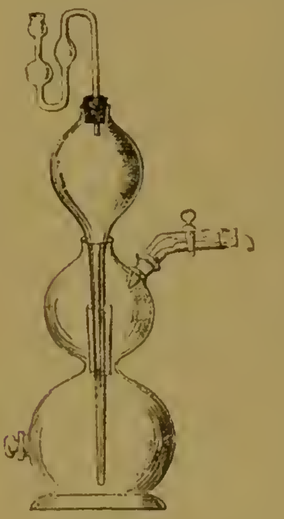

FIG. 119.—KIPP'S APPARATUS, FOR GENERATING HYDROGEN AND OTHER GASES

The gas coming through the exit-tubing is led under water, and collected, after five minutes, in an inverted test-tube full of water. It is tested in the usual way, the test-tube—mouth downwards, and now full of gas—being plugged with the thumb, and its mouth then opened close to a small flame. If the gas burns quite quietly, all the oxygen in the tube of medium may be considered to have been replaced by hydrogen. The gas should then be passed in for five minutes more, and the piece of *exit-tubing* (*b*) fused, as in the illustration. The gas is still passed in so as to completely fill the culture tube, and then the *entering-tube* is quickly fused.

An Esmarch's roll-tube culture is then made (p. 21), and incubated at the appropriate temperature. Colonies of the anaërobe then develop in the layer of gelatine, or agar, which forms a lining to the glass tube.

An objection to the use of gelatine, or agar, in this way is that owing to its viscidity large bubbles are produced in passing in the hydrogen, and these subsiding but slowly, or not at all, materially interfere with the practical employment of this method. It has therefore been recommended *to make a roll-tube first*, in the ordinary way, and pass the hydrogen in subsequently. This drives out all the oxygen equally effectually.

---

[1] The indiarubber stopper and tubes are freshly sterilised and kept in the steamer (fig. 2), until ready to be substituted for the wool plug, as described.

A *modification of this second method* consists in making a roll tube, solidifying it in water containing ice, and pouring in liquefied gelatine, or agar, which then, after driving out all the oxygen, forms a solid core. After replacing the wool plug, the tube is fitted with an indiarubber cap in the usual way. The advantage of this method over a stab- or a shake-culture is that the colonies are developed next to the side of the tube, and not in the depths of the medium, so that they can be readily observed.

In studying the chemical products of anaërobes, nutrient broth must be used and Fränkel's method may be adopted. For this purpose, indeed, broth is more convenient to work with than liquefied gelatine, or agar. The method of passing the hydrogen through the inoculated broth, and then sealing up the pieces of tubing, is exactly the same as when liquefied gelatine, or agar, is used.

For class work, the inoculation of **stab cultures of 1 per cent. glucose agar** (fig. 121), or of **0·5 per cent. formate of sodium agar-agar,** is perhaps the most suitable method of growing the anaërobes. After inoculation, contact with the air can be further cut off by covering the surface of the medium to a depth of about half an inch with formate of soda solution, or with petroleum, sterile oil, &c.

Streak formate-agar cultures, similarly covered with fluid, may also be tried.[1] **Formate broth** sometimes gives good results.

One per cent. glucose gelatine, carefully neutralised, or having a slightly alkaline reaction, may also be employed.

Shake cultures of glucose-agar, and -gelatine (fig. 120), should be used to demonstrate the formation of gas, and the specific characters of the colonies, in the case of the three anaërobes we are about to study.

In all cases, the medium—whether gelatine, agar, or broth—*should be freshly prepared*, immediately before use. This is especially important in the case of tetanus cultures. The tubes are capped and then kept at the appropriate temperature, 22° C., or 37° C.

A very convenient method of examining the cultures by means of the low power of the microscope is afforded by making **plate** (petri-dish) **cultivations,** with the above-mentioned media, in an atmosphere of hydrogen. The plates are made in the usual way, but special petri-dishes have to be employed, so that the hydrogen may be passed through, and the anaërobes kept constantly in an atmosphere of that gas.

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[1] For film preparations, especially for flagella staining, in the case of tetanus, Kanthack used **streak cultures of formate-of-soda-agar, grown in Buchner's tubes.** (See p. 222.)

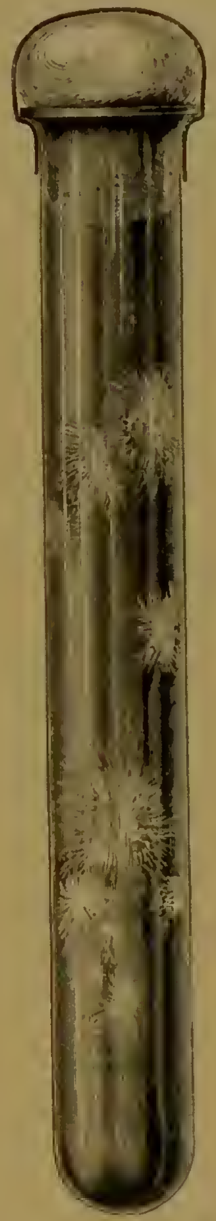

Fig. 120. — ANAËROBIC GELATINE SHAKE CULTURE OF THE TETANUS BACILLUS

(From Schenk's 'Bacteriology,' after Frankel and Pfeiffer.)

Botkin's apparatus[1] for growing anaërobes is extremely simple. A bell jar rests in a glass dish containing petroleum, or some similar fluid, upon a leaden support, so as to allow two bent leaden tubes to pass down between the side of the dish and the bell jar and then up to just above the free surface of the fluid, which cuts off contact with the outer air. Hydrogen is passed in, until the bell jar is completely full of the pure gas. Culture tubes, plates, &c., previously inoculated, are placed on appropriate supports under the bell jar, and the apparatus kept at a suitable temperature.

Tetanus

If rabbits, guinea-pigs, or mice, be inoculated subcutaneously with ordinary garden earth, death frequently results from tetanus in from twenty-four to forty-eight hours. At the seat of inoculation the tissues are swollen, yellowish, and œdematous, this condition spreading into the parts around. There is generally a localised abscess, the pus containing numerous other organisms besides tetanus bacilli which may be isolated after inoculating agar-agar tubes with the pus at 37° C., in the following way.

To isolate the tetanus bacillus, from such a culture.—Film preparations are made; and if the drumsticks, typical of spore-bearing tetanus bacilli, are seen after the tube has been inoculated altogether for forty-eight hours, the culture is heated in a water bath at 80° C. for one hour, to destroy the less resistent contaminating bacilli, according to **Kitasato's method.**

Roll-tubes are now made of nutrient gelatine, using as little of the medium as possible. Hydrogen is passed in one of the ways already described, and the tube hermetically sealed. The growth appears at the end of a week, the tube being kept at from 18° to 20° C. At the end of ten days, the colonies may be examined with the microscope, and pure sub-cultures made. From such pure cultures, susceptible animals—guinea-pigs, mice, or rabbits—should be inoculated to test the virulence of the cultivations. The tetanus bacillus is not always an easy anaërobe to grow. The reaction of the medium should be examined immediately before use, and should be only slightly alkaline. If using nutrient broth, it may be advisable to neutralise it by the addition of 10 c.c., or so, of a 1 per cent. solution of sodium hydrate to every litre of the broth in question.

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[1] A figure illustrating the apparatus will be found in the English edition of Thoinot and Masselin's *Outlines of Bacteriology*.

Cultivations.—*Glucose-gelatine stab.*—The tetanus bacillus is an obligate anaërobe. The growth begins at a distance of three quarters, to one inch, or more, below the surface of the medium. If kept at 18° to 20° C., it does not usually appear under a week. At 22° to 24° C., however, it may appear in from three to four days. The growth along the track of the needle is somewhat hazy, and lateral branches radiate out into the surrounding medium. The gelatine is only very slowly liquefied, and some gas may sometimes be produced. If present, this would best be seen in shake cultures (fig. 120). The young colonies are opaque white in the centre, and a radiating fringe of fine filaments, not unlike those of young favus colonies, is seen at the periphery. In old colonies, the appearance presented somewhat resembles the felted surface of a common white mould.

Glucose-agar stab.—The growth takes place best at from 37° to 38° C. At the end of forty-eight hours, there is a distinct growth along the track of the needle, from which very delicate, branching processes, composed of fine, white, punctiform colonies, spread into the surrounding medium, so as to result, eventually, in a very characteristic tree-like growth (see fig. 121, A). Little, or no, gas is formed, the appearance of the growth, and the very slow development, or absence, of gas readily distinguishing tetanus from the two succeeding organisms.

Glucose broth.—In an atmosphere of hydrogen the organism grows readily, especially if the broth be fresh, and rendered slightly alkaline, in the manner directed above. The addition of 1 per cent. of glucose is of considerable value; or if 0·5 per cent. of formate of soda be added to the broth, it is not necessary to grow in an atmosphere of hydrogen provided the tube be carefully and completely sealed by means of the indiarubber cap. The medium is rendered at first slightly turbid; but in three or four days there is a white deposit of from $\frac{1}{16}$ to $\frac{1}{8}$ of an inch deep at the bottom of the tube, the liquid above being of a clear straw colour: like the cultures in all the other media, there is a very fœtid odour, and gas in small quantity may be developed.

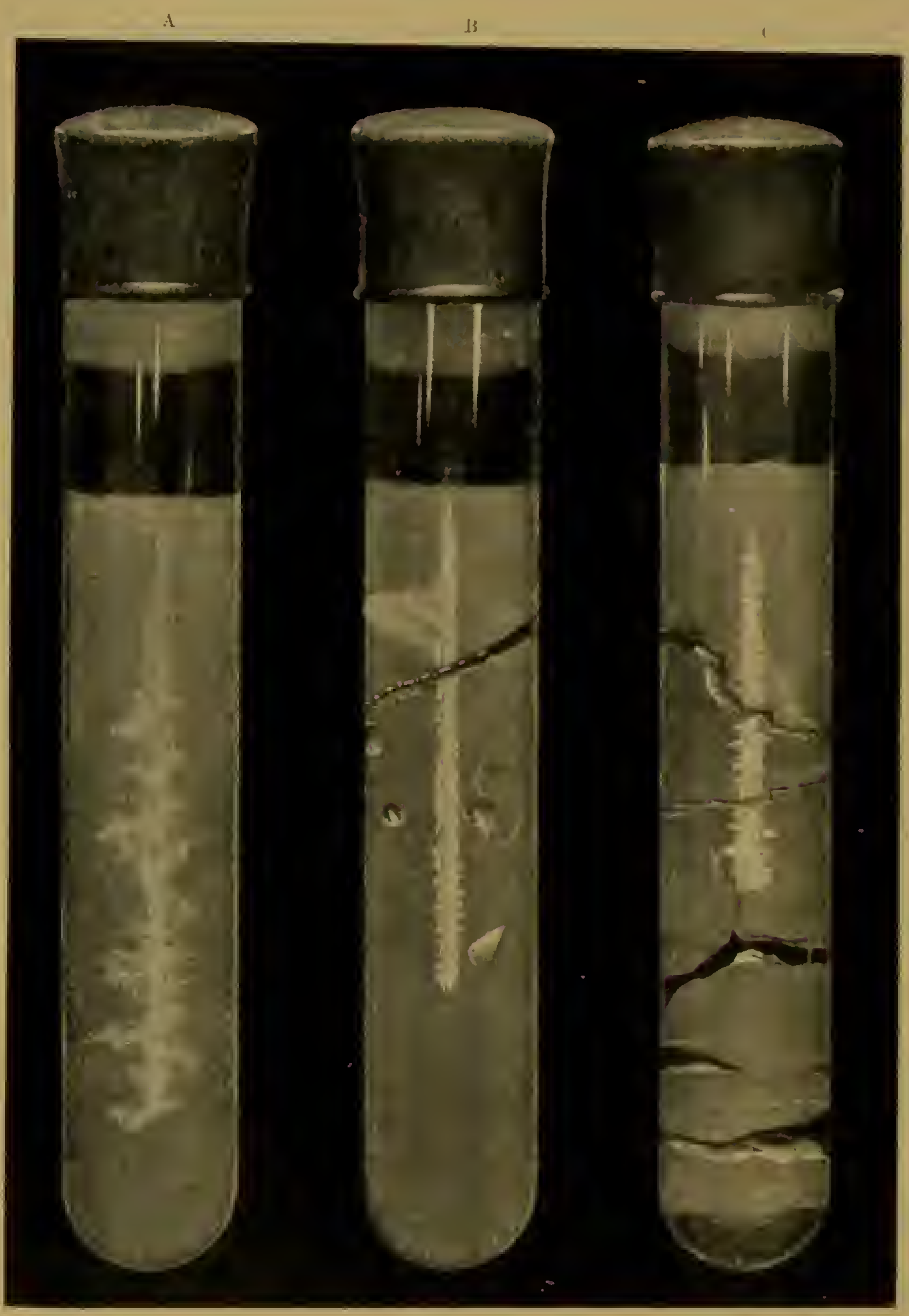

Fig. 121

A, TETANUS BACILLUS
Glucose-agar culture, five months old.

B, BACILLUS OF MALIGNANT ŒDEMA
Glucose-agar culture, two days old.

C, BACILLUS OF QUARTER EVIL (SYMPTOMATIC ANTHRAX)
Glucose-agar culture, two days old.

Coverslip preparations are best made from surface cultivations on formate-of-soda-agar grown in Buchner's tubes, as described by Kanthack and Connell.[1] This method is especially advisable

[1] *Trans. Path. Soc. Lond.* vol. xlviii. 1897.

when the flagella are to be examined, the objection to the use of stab-cultures being the unavoidable mixture of the organisms with the medium itself. The films for flagella staining may be made from such cultures: (*a*) when four days old, and (*b*) when fourteen days old.

Under the microscope, tetanus bacilli are seen to be rods of varying length, some of them being short and stumpy, others long and thin; sometimes threads of considerable length are seen, and in these forms the width may be less than in the shorter ones, although this is not invariably the case. When the spores have formed, the bacilli have the very characteristic appearance of drumsticks, the spore itself being spherical and highly refractile. It is usually terminal, and by its circular outline, and generally larger diameter, may be distinguished without difficulty from the somewhat similar spore-bearing bacillus of Quarter Evil.

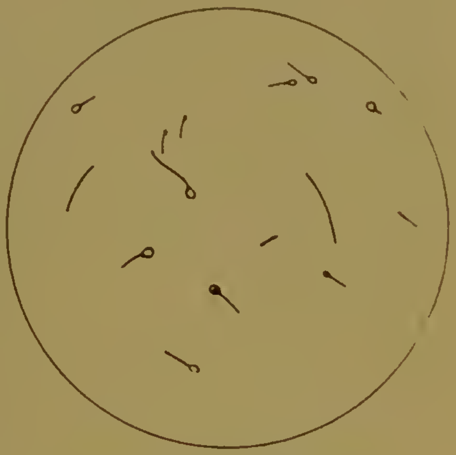

FIG. 122.—TETANUS BACILLI AND SPORES. × 530

Motility.—The organism is only slightly motile,[1] and to demonstrate its motility, the use of a warm stage is advisable.

Flagella.—The tetanus bacillus is provided with numerous flagella, sometimes twenty to thirty, a point to which the late Professor Kanthack and Connell have drawn attention in a well-illustrated paper,[2] from which the following account is taken. Some of the flagella are thicker than others, and they are either single, or consist of numerous flagella twisted together. The more numerous and finer flagella are distinguished as *primary*; the thicker, and more or less spirally twisted flagella, just mentioned, of which there may be from one to three to each bacillus, are considered to be *secondary*. Even the long thread-like bacilli may have a few fine flagella.

After the formation of spores the organism loses its motility, and the fine primary flagella disappear first, so that the thick secondary flagella—which may be single, or form a terminal tuft—alone remain visible. They are readily demonstrated by Van Ermenghem's, or Pitfield's, method.

Spore formation.—In nutrient gelatine, which can only be incubated at, or below, 24° C., spore formation does not usually appear before a week, by which time considerable liquefaction of the gelatine may have occurred. Kept at the more favourable temperature for tetanus, 37° to 38° C., spore formation occurs in agar, broth, &c. in about thirty hours.

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[1] The slight motility, in spite of the numerous flagella, in the case of tetanus bacilli, has been attributed chiefly to the unfavourable, *aërobic* conditions of examination—i.e. the hanging-drop, as usually made.

[2] *Loc. cit.*

Vitality and virulence of tetanus spores.—When sterilised threads (p. 267) are placed in a broth culture of the tetanus bacillus, and dried in an ordinary desiccator containing concentrated sulphuric acid, the spores remain alive, and retain their virulence, for many months. They are not killed after exposure to 80° C. for an hour; but, if kept in the steam steriliser at 100° C., they are killed in five minutes.

The solution obtained by adding 0·5 per cent. of hydrochloric acid to a 1 in 20 solution of carbolic acid, or to a 1 in 1,000 solution of corrosive sublimate, is seven or eight times more rapid in its action in destroying the spores than these solutions unmixed with the hydrochloric acid.

In the case of exposure to a solution of 1 in 20 carbolic acid alone, the spores survive for ten hours, but are destroyed in fifteen hours: when the hydrochloric acid is added in the proportion mentioned, two hours' exposure is fatal. In the case of a 1 in 1,000 solution corrosive sublimate, three hours are necessary; but only half an hour, when 0·5 per cent. of hydrochloric acid is added to the mixture (Abbott).

Inoculation of pure cultures subcutaneously.—Mice, rats, guinea-pigs, rabbits, and other animals are readily susceptible to tetanus, and after a variable period—which is about twenty-four to thirty-six hours for mice, and longer for the larger animals mentioned—tetanic spasms occur, commencing at the seat of inoculation and soon becoming general. As in the case of a frog into which strychnine has been injected, these spasms are readily excited, either by gently touching the animal, or by merely blowing on it. Death is caused by the manufacture of the toxic products at the seat of inoculation of the organism, which, however, is often extremely difficult to find, a hyperæmic condition at the seat of the injection being often all that is visible when the culture is pure. If earth, or other material containing the organism, is used instead of a pure culture, there is commonly a focus of suppuration in the subcutaneous tissue into which the inoculation has been made, and the characteristic drumsticks, or free spores, may be found in the pus. There appears to be no evidence of the multiplication of the organism in the tissues, the organs of animals that have died of tetanus failing to produce the disease after an inoculation of susceptible animals, though the blood, pleuritic effusion, &c., containing the toxic products, will produce the disease.

Tetanus, like diphtheria, can be experimentally produced by the filtrate of broth cultures containing toxic products only, the bacilli being separated off by the Pasteur-Chamberland filter. The virulence of such toxic products is truly remarkable, and may be so great that $\frac{1}{20}$ *millionth of a gramme*, or 0·00005 *milligramme*, is quite enough to kill a small mouse.

Death is due, in fact, to a **toxæmia**, as in the case of diphtheria.

Tetanine, *tetano-toxin*, and *spasmo-toxin* are amongst the bodies isolated from fluid cultures of the organism. They are similar in chemical nature to those obtained in the case of diphtheria.

How the tetanus toxin enters the system.—Tetanus toxin reaches the central nervous system from the point of injection along two paths: one portion directly infects the nerves, accounting for the localised contractions of muscle sometimes seen; the other spreading by the blood. The cells of the central nervous system show a strong affinity for the toxin, those of the spinal cord being saturated before the higher nerve centres are attacked.[1] Wassermann subcutaneously injected a mixture of tetanus toxin and an emulsion of brain, or spinal cord, into guinea-pigs, and no bad symptoms occurred; whereas, the control animals treated with the toxin alone died of tetanus.

Protective and curative serums.—Experimentally, susceptible animals may be protected from the lethal effects of very large doses of the tetanus organism and its products by a series of sub-lethal doses, so that eventually they not only become immune themselves, but the serum derived from their blood acts as a protective and curative agent for other animals. In the first case the immunity is active, in the last-mentioned animals it is passive (see pp. 166–7). The passive immunity, acquired by hypodermic injection of serum from an animal already immunised, only holds good, however, against tetanus toxin hypodermically or intravenously injected, not for intracerebral injections of the toxin, in which case cerebral tetanus is set up; so that, clearly, the brain cells are not rendered immune by a subcutaneous or intravenous injection of tetanus anti-toxin.

This explains the experimental fact that, whilst subcutaneous or intravenous injections of the anti-toxin may fail to cure tetanus produced in animals, intracerebral injections in the early stage of the disease are most successful, as shown by Roux and Borrel,[2] and they have applied this method to the treatment of man. Major Semple (until recently of Netley), to whose courtesy I am indebted for a demonstration of the mode of operating, and from whom the foregoing account[1] has been taken, has verified the efficacy of this method, and records a successful case, several others being reported subsequently during the same year (in 1899).

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[1] After inoculating rodents with tetanus-toxin, and hardening the nervous systems in formalin, Foulerton and Campbell Thomson (*Lancet*, January 13, 1900) have actually succeeded in demonstrating, by a modification of Nissl's process with methylene-blue, *definite changes in the constitution of the nerve-cells* of the motor areas of the cerebral cortex, &c., and to these may be attributed the motor disturbances occurring in tetanus. For Nissl's method, refer to von Kahlden's *Pathological Histology*, p. 136.

[2] 'Tétanos cérébral et Immunité contre le Tétanos,' *Annales de l'Inst. Pasteur*, April 1898.

Description of the operation for the intracerebral injection of anti-toxin in tetanus.

The line of the incision is planned so as to allow of the nozzle of a syringe to enter the brain in front of the motor areas. From the mid-point of an imaginary line joining the two External Auditory meatuses, a line is drawn to the outer angle of the orbit. Through the mid-point of this second line the ½ to ¾ inch incision is made, and the bone just perforated by an Archimedean drill, which need be little larger than the needle of the syringe. The needle, which is rounded at its point to lessen the danger of transfixing a vessel and so causing hæmorrhage, is two inches long and is plunged into the substance of the brain as deep as it will go. The syringe has a screw-piston and is connected with the needle by three inches of tubing. The anti-toxin used is double the strength of the ordinary anti-toxin, and 2½ c.cm. are inserted into each cerebral hemisphere, the injection being slowly performed, drop by drop, allowing at least ten minutes for each injection.

'In addition to this, the patient receives 20 c.cm. anti-toxin hypodermically, for two, three, or four days, according to circumstances. The intracerebral injection immunises the higher nerve centres before the toxin has been fixed there. The anti-toxin given hypodermically renders the blood anti-toxic, and the toxin, as it becomes absorbed from the source of supply—wound, bruise, or any other source—is neutralised as soon as it enters the blood' (Semple). Mr. H. S. Collier, in March 1899,[2] performed, at the suggestion of Mr. Plimmer, **subdural injection** through a small trephine hole made over the back of the cerebellum, with the idea of bringing the anti-toxin, thus mixed with the cerebro-spinal fluid, more rapidly and directly in contact with the vital centres in the medulla. The anti-toxin was also injected hypodermically. His success in one case, at St. Mary's Hospital, London, was soon afterwards repeated by a colleague, in a second case. Mr. Horsley, at University College Hospital, in May 1900, was similarly successful. The original wound, situated in the scalp, was freely excised. *This should be done in all cases,* so as to remove the source of infection.

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[1] 'The Treatment of Tetanus by the Intracerebral Injection of Anti-toxin.' *Brit. Med. Jour.*, January 7, 1899.

[2] *Lancet*, May 13, 1899.

Malignant Œdema, and the 'Vibrion Septique'

Malignant œdema, or **septicæmia of Pasteur,** like tetanus, is sometimes the result of inoculation with ordinary garden earth, street dust, &c. It affects man, horses, sheep, dogs, and fowls (slightly), rabbits, guinea-pigs, and mice, the last-mentioned animal being one of the most susceptible of all, dying, as the result of inoculation, sometimes in less than twenty-four hours, with the specific organism in the blood in small numbers. The bacillus of malignant œdema, called by its discoverer, Pasteur, '*vibrion septique,*' appears to be present in most soils, and in the intestines of many animals, in these respects, as well as in that of the class of animals affected, differing considerably from the bacillus of Quarter Evil (*q.v.*).

The characteristic lesion is a well-marked œdema, from which the organism can readily be isolated; and a condition of surgical emphysema, from the production of gas in the tissues. Spreading traumatic gangrene, emphysematous gangrene, or gangrenous septicæmia, as it is called by the French, is due in many cases to this organism, though other bacilli, e.g., *B. coli*, and *B. aërogenes capsulatus* (p. 104), have been found in such lesions.

Cultivations.—The bacillus of malignant œdema is an obligate anaërobe, and must therefore be cultivated out of contact with oxygen.

Glucose-gelatine stab.—The growth occurs along the track of the needle at a distance of about an inch, or an inch and a quarter, from the surface, and the gelatine is liquefied, with the formation of gas bubbles.

Glucose-gelatine shake cultures (fig. 123) demonstrate the properties of this organism very well, small transparent globes of liquefied

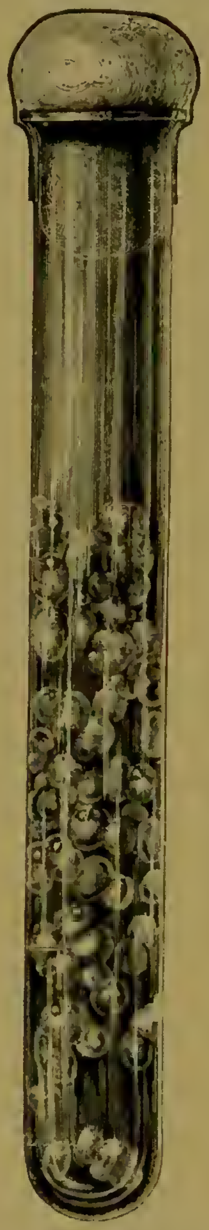

Fig. 123.—Bacillus of malignant œdema

Shake glucose-gelatine culture. (From Schenk's 'Bacteriology.')

gelatine being formed near the bottom of the tube. Later, the transparency is replaced by a cloudiness, from which fine striæ, visible with a lens, pass into the surrounding medium (Abbott).

Glucose-agar-agar stab.—The growth along the track of the needle is white, and is thicker and more developed below than above (fig. 121, B). The edge of the growth consists of discrete, dotted colonies, which are seen spreading into the medium, but they do not produce the delicate tree-like growth seen in the case of tetanus. Gas formation occurs rapidly at 38° C., and the medium rapidly cracks on this account. Cultivations have a fœtid odour.

Glucose-agar streak.—Surface cultures may be made on glucose-agar, and included in larger tubes containing pyrogallic acid and sodium hydrate, according to Buchner's method. This is convenient for making clear film preparations unmixed with medium. Formate of sodium agar-agar may also be used, streak cultures growing well, as in the case of aërobes, if the tube be sealed by means of a rubber cap, no pyrogallate of sodium being necessary. (See footnote to p. 225.)

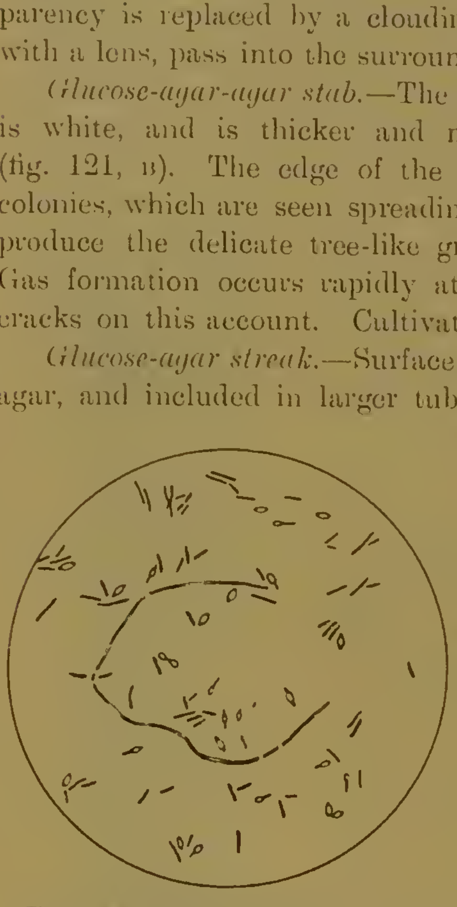

FIG. 124.—BACILLUS OF MALIGNANT ŒDEMA. × 530

Formate-of-sodium-broth, or *glucose-broth.*—In the case of glucose-broth, especially, hydrogen should be passed in. Growth occurs rapidly, the broth being at first rendered turbid, but subsequently becoming clearer, from the deposition of the white growth at the bottom of the tube. Gas formation (CO_2, and H) results, producing the characteristic fœtid odour.

Under the microscope, the organism is seen to be a slender rod of varying length, there being frequently single bacilli with rounded extremities, which are, generally speaking, about the length of an average *B. anthracis*. Frequently, however, long threads are seen, which form segments of unequal length; the extremities of these segments, where apposed, are square, or bevelled off obliquely.

Motility.—The bacillus is slightly motile.

Flagella are present, according to Abbott, at the ends, and along the sides.

Spore formation rapidly occurs, the appearance of the spores being preceded by a swelling in the middle of the bacillus, so that this eventually becomes spindle-shaped, the highly refractile oval spore occupying the greater portion of the bacillus, which in this condition is only coloured at its polar extremities, if the simple stains are used.

Inoculation with soil.—It is advisable to insert the soil into a pocket made in the subcutaneous tissue; so in the case of pure cultures, large quantities should be used; or bits of the tissue, or some of the œdematous fluid of an animal which has died of the disease, should be inserted in the subcutaneous layers, through an incision in the skin of the abdominal wall or thigh.

Susceptible animals, such as mice, may die in from twenty-four to forty-eight hours, with widespread, more or less blood-stained, œdematous tissues, from which the specific organism may be readily obtained. The muscles have a blackish appearance, the spleen is soft, the liver dark, and the lungs may look either pale, or discoloured (Pasteur).

In the case of mice, the heart blood is said to contain the organism at the time of death; whilst in the case of other animals, rabbits, &c., this does not occur until some hours after death, although they may be found in the viscera themselves, and in effusions into the pleural and peritoneal cavities, in which there may be a very considerable amount of exudation, possessing an extremely fœtid odour.

Symptomatic Anthrax, or Quarter Evil (Charbon Symptomatique; Rauschbrand); and Chauveau's Bacillus

Symptomatic Anthrax, or Quarter Evil.—This disease is also known as 'black leg' or 'quarter ill.' It affects oxen from six months to four years old; never older or younger, it is said. It is to be noted that swine, dogs, rabbits, and fowls, which are readily susceptible to malignant œdema, are absolutely refractory to spontaneous symptomatic anthrax. Horses, while readily susceptible to malignant œdema, naturally and experimentally, are only locally, and not seriously, infected by the organism of symptomatic anthrax (Abbott). Experimentally, the only animals *not* immune are sheep, goats, oxen, and guinea-pigs.

Contrasting with what has been said of the bacillus of malignant œdema, virgin soil, generally, does not appear to contain the organism. Diseased cattle, however, especially after death, infect the soil in the neighbourhood, which is thus a constant source of danger.

Symptomatic anthrax resembles true anthrax in but slight degree. It is characterised by movable, emphysematous swellings of the subcutaneous and deeper muscular structures over the quarters of the hind limbs, the tissues affected being almost black in colour, œdematous, and containing gas. The black, gangrenous, appearance has given rise to the common English name of 'black leg.' The organism is found in large numbers in this diseased area, and also in the blood-stained serous exudations, in the pleural and peritoneal cavities, in the bile, the liver, and the viscera generally, and in the lymphatic glands, which may be deeply congested, or exude a large quantity of yellow serum. The blood at death may contain only a few bacilli. They multiply rapidly if the blood is incubated for twenty-four hours at 37° C. As a rule, no spore formation occurs in the tissues during life; but, in less than twenty-four hours' time after death, typical spore-bearing bacilli may be found. The organisms in the tissues are generally arranged singly, occasionally in pairs. It is hardly ever seen, even in cultures, in long threads, as in the case of malignant œdema.

Cultivations.—The cultures, whether liquid or solid, have a peculiar rancid odour.

Alkaline broth, containing 1 *p.c. of gelatine* and 1 *p.c. of glucose*, has been recommended as a favourable medium. It should be kept at 36° to 38° C.

Liquid serum, with, or without, the addition of water, is said to be still better. In either case, the oxygen must, of course, be excluded by the passage of a current of hydrogen, as usual.

Sodium-formate broth, 0·5 p.c., may also be used, as in the case of the other anaërobes.

Whichever medium be used, in from twenty-four to forty-eight hours, turbidity is noted, and gas bubbles are seen on the surface of the liquid; a white flocculent deposit sinks to the bottom of the tube.

Glucose-gelatine stab.—After forty-eight hours, or so, a growth appears about an inch below the surface. It is white, and liquefaction of the medium, with gas formation, occurs after a time.

Glucose-gelatine 'shake.'—The colonies appear, in the lower two-thirds of the medium, as tiny spheres rapidly liquefying the gelatine, two or more of such adjacent liquefying colonies often coalescing into one.

Glucose-agar stab.—This is an excellent medium, the growth appearing in from thirty-four to forty-eight hours, at about three-quarters of an inch below the surface (fig. 121, c). The colonies along the track of the needle form a somewhat denser mass than in the case of malignant œdema, which it, however, closely resembles. The lower end of the growth is broader than the pointed upper extremity; and from its margins, minute, rounded, or slightly oval, colonies begin to sprout out into the surrounding medium. There is, however, no branching tree-like growth as in the case of tetanus.

Gas forms even more rapidly and more abundantly than in the case of malignant œdema, and its production is almost invariably associated with more or less horizontal splitting of the medium. This gas formation sometimes proceeds so rapidly as to force the upper layers of the medium against the wool plug, and any fluid present may actually be driven through the wool, the partially liquefied medium running down outside the tube.

Sodium-formate (0·5 *p.c.*) *agar stab, or streak.*—In the case of the streak culture, merely capping the tube tightly may be sufficient, but it may also be inserted in a larger tube containing pyrogallic acid and sodium hydrate, according to Buchner's method. This is the most convenient method for making coverslip preparations, especially when it is desired to demonstrate the flagella.

Under the microscope, the organism appears as a slender rod with rounded ends, and may be slightly curved, or sinuous (fig. 125). It occurs singly, or in pairs; but, as already mentioned, the very long thread-like forms, commonly seen in the case of malignant œdema, are not as a rule observed in the case of symptomatic anthrax. It is decolorised by Gram's method.

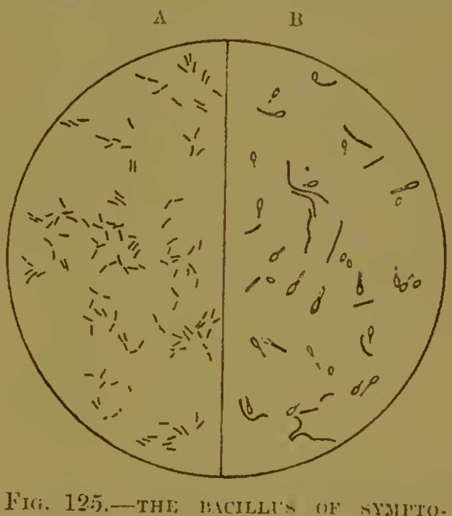

Fig. 125.—THE BACILLUS OF SYMPTOMATIC ANTHRAX, OR CHAUVEAU'S BACILLUS. × 530

A. young culture. B. spore-bearing organisms.

Sections may be stained by Löffler's blue; or better, perhaps, by Kühne's carbol-methylene-blue.

The spores are generally terminal and give the bacillus a very characteristic appearance. Spore-bearing rods may also be seen, in tissues, especially between the fibres of the muscles in the affected areas. Transverse fractures of the fibres, with bacilli lying in the gaps, are also not uncommonly seen (Thoinot and Masselin). The spore itself is elliptical, and somewhat flattened laterally, and has a pointed free extremity. It is sometimes situated in the middle of the bacillus, when it is not infrequently mistaken for the bacillus of malignant œdema, which, however, is a larger organism. The absence of long threads is a marked feature in symptomatic anthrax, and the bacilli are more motile; also, in the case of animals dying from the disease, spore formation is found to occur more readily in the tissues than in the case of malignant œdema.

Motility.—Before spore formation has occurred, the organism is very motile, much more so than in the case of malignant œdema.

Flagella.—The organism possesses numerous flagella.

Spore formation occurs in from thirty-six to forty-eight hours on media kept at the body temperature; but in the case of gelatine kept at 22° C. this is delayed for about a week. The spores, generally speaking, appear to be as resistent to the action of heat and chemicals, corrosive sublimate, carbolic acid, &c., as in the case of tetanus.

Inoculation.—Cultures rapidly lose their virulence, and for inoculation purposes the safest material is the blood-stained, œdematous fluid, or portions of the diseased tissues, inserted into a pocket beneath the skin. The best animal for such inoculations is the guinea-pig.

Protective inoculation.—One attack of the disease, if survived, confers immunity. Exudation from the swelling in the diseased muscle has been injected in small quantity into cattle, and on recovery the animals have been found immune against a much stronger dose.

In practice, to quote Crookshank, this exudation and diseased muscle are dried at 32° to 35° C., made into a powder, and then mixed with water. This infusion is heated to 100° C., and forms the first vaccine. After ten days a second vaccine, prepared in the same way and heated to 80° C., instead of 100° C., is injected, as before, into the under surface of the tail, near its extremity. For general use these vaccines may be kept in the form of dry powder, one-tenth of a gramme of which is gradually rubbed up with 5 cc. of water, $\frac{1}{2}$ cc. being used as a dose for each animal. Complete success results from the use of the second vaccine.

Other methods of immunising animals have been suggested, Roux and Chamberland using filtered cultures; Kitasato employs old agar cultures, or fresh cultures sterilised by steam for thirty minutes. Crookshank[1] brings forward evidence tending to prove that the efficacy and safety of protective inoculation are not fully established.

There is sometimes a difficulty in distinguishing, *microscopically*, *B. anthracis* from the organism of symptomatic anthrax. The following table may therefore be useful:—

Anthrax	*Symptomatic Anthrax* (*Rauschbrand*)
Cultures.—Mainly aërobic, but typical fir-tree growth occurs in stab gelatine, with liquefaction of the medium. The cultures have no odour. The organism is non-motile.	The organism of quarter evil is strictly anaërobic, and will not grow in contact with the air. The cultures have a characteristic rancid odour. The organism is actively motile, until the occurrence of spore formation.

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[1] *Bacteriology and Infective Disease*, pp. 219, 220.

Anthrax	*Symptomatic Anthrax* (*Rauschbrand*)
Film preparations.—The bacilli vary in length from 2μ, or 3μ, to 20μ, or even 35μ; frequently very long threads are seen, when the organisms are obtained direct from the tissues, or blood, of the animal. The bacilli are seen to be short rods, more or less square at the ends. When carefully examined under high magnification, the organism is not infrequently seen to be slightly thicker at the ends than in the rest of its length, and its extremity is slightly concave,[1] so that two apposed bacilli have a somewhat knotted, bamboo-like appearance. The spores, being central, not terminal, do not produce a paddle- or club-shaped appearance; and, in the tissues of the living animal, spore formation is never found.	The bacilli have rounded extremities: they are often single, and occasionally paired, but have no tendency to form very long threads, though sometimes, they are considerably curved. The spores are frequently formed at, or near, one extremity, when the bacillus has a somewhat paddle-shaped appearance. Spore formation, though not always present immediately after death in the tissues of the animal, readily occurs in from 20 to 24 hours.
The organism is stained by Gram's method.	The organism is decolorised by Gram's method.

LESSON XXI

MALARIA AND BLACKWATER FEVER. AMŒBIC DYSENTERY. PSOROSPERMOSIS. PAGET'S DISEASE OF THE NIPPLE. CANCER

MAKE film preparations of blood from a patient suffering from an attack of Ague, as follows:[2]—

A. Fresh preparations.—(i) Carefully cleaned coverslips are fixed in Cornet forceps and kept at hand.

(ii) The pulp of one of the patient's finger-tips (the skin of the ear is recommended by some) is carefully cleansed with, successively, soap and water, turpentine, alcohol, and ether, the two last-mentioned being merely poured on. The skin is allowed to dry by evaporation of the ether. The cleansed finger is held between the investigator's left index finger and thumb, the pressure of the latter causing the pulp of the finger to become congested, so that the slightest stab with a needle—previously sterilised in 5 p.c. carbolic acid, and then placed in boiled water—causes the blood to appear. There is no need to wind a tape round the finger, as a rule.

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[1] See, however, p. 136.

[2] If malaria blood is not obtainable, these methods of preparing, fixing, and staining films may, with advantage, be practised with normal blood.

The first drop of blood which appears is wiped off, and when a *very minute* second drop has been squeezed out, the coverslip is applied to it, and at once placed on a clean slide.

Prolonged search with a $\frac{1}{12}$-in. oil-immersion is to be made for the *Plasmodium malariæ*, frequently pigmented and actively motile, inside the red corpuscle. If such parasites are found, it is desirable to make a hanging-drop preparation in the way recommended by Mason (see below) from a fresh drop of blood, so as to study the development of *flagella*, which only occurs after the blood has been shed for at least twenty minutes.

B. Permanent preparations.—(I.) *Prepare films* for staining in one of the following ways:—(i) The drop of blood is collected on a coverslip as above. A second coverslip is applied to this, and a thin film is produced by rapidly drawing them apart. The coverslips are dried by waving rapidly, film side down, in the air.

(ii) Collect a small drop of blood at one end of a clean glass slide. This may be spread with the edge of a clean coverslip, which is inclined at an angle of about 45° to the slide and rapidly drawn across it from end to end.

Hyem prefers to use, instead of the coverslip, a thin glass rod, which is swept rapidly along the slide, so that the small drop of blood at one end is spread out into a uniformly thin layer over the whole slide.

Dry in the way just described. Fixation is accomplished by one of the methods to be mentioned.

(iii) *Manson's method* gives excellent results, and consists in collecting a drop of blood on a piece of thin tissue paper (*e.g.* cigarette paper). This is then laid down near one edge of a clean coverslip. The paper is allowed to come into contact with the glass, and the blood spreads by capillary attraction for some distance over the surface. A uniformly thin layer is now produced if the paper—whilst kept practically in the same plane as the glass—is drawn along with a sliding movement. A series of films should now be made from the *same piece of paper*, similarly applied and drawn along other coverslips placed ready for use.

Dry. Fix, in one of the ways now to be described.

(II.) **Methods of fixation.**—Practise each of the following methods, and note which gives the best results.

(*a*) Pass the dried coverslip three times rapidly through the flame.

(*b*) Expose the coverslip, film side down, to the open mouth of a bottle containing 1 per cent. osmic acid solution for two minutes; this is an excellent method, preferred by Hyem.

(*c*) Place the dried coverslip in a closed jar, containing equal parts of absolute alcohol and ether, till required for use.

(*d*) Place moist film preparations, for half an hour or more, in a saturated solution of corrosive sublimate, made with boiling normal salt solution. Subsequent prolonged washing in normal salt solution water is necessary in this case.

(*e*) Keep the film in a hot-air chamber at 120° C. to 140° C. for fifteen to thirty minutes. This is one of the best methods.

Where **fresh blood** can be examined from a patient it is not necessary to stain, the characteristic, generally pigmented, and frequently very motile, plasmodial body being remarkably distinct even in the unstained condition. Use the $\frac{1}{12}$-in. oil-immersion lens for the examination. In India, a $\frac{1}{20}$-in. water-immersion lens is commonly used. No specimen should be passed by without at least half an hour's careful search, according to Manson.

(III). **Methods of staining.**—Films may be **stained,** after fixation as above, in each of the following ways:—

(i) Use a saturated watery solution of methylene blue for five minutes, and wash well. Dry and mount.

(ii) Stain with a 1 per cent. watery solution of eosin for five minutes, wash thoroughly, and then expose to saturated watery methylene blue for two to three minutes. Wash well, dry, and mount. This gives excellent results; the red blood corpuscles and the eosinophile and basophile granules are stained pink, the nuclei of the white corpuscles are stained blue, the plasmodia in the red corpuscles being also stained blue. The plasmodia are distinguished from nuclei by their shape, and by the presence of pigment, generally.

(iii) **Jenner's combined eosin and methylene-blue stain.**—After one to two minutes' exposure to the stain, wash till a pink tint appears. Dry and mount. This rapid method gives quite good results.

(iv) **Ehrlich-Biondi solution.**—Exposure for half an hour, with subsequent rinsing, gives very pretty results, Muir and Ritchie recommending the specimen to be examined without previous mounting in Canada balsam. (To stain sections, see p. 257.) The nuclei are stained a greenish-blue, the oxyphile granules red, the so-called 'neutrophiles' purple, the basophiles being unstained.

(v) **Ehrlich's hæmatoxylin.**—Expose for one minute; wash well in distilled water; place in a 2 per cent. solution of acetic acid for three-quarters to one minute, and then thoroughly wash in tap water. It may be left in this for half an hour or more without harm. Counter-stain with 1 per cent. watery eosin for one minute. After thoroughly washing the coverslip, it is dried and mounted as usual.

All nuclei and plasmodia are stained blue.

Malaria and the Plasmodium Malariæ

The plasmodium malariæ.—If the blood of a patient suffering from a fit of ague be examined, unstained, just before, or during, the stage of rigor, a variety of appearances may be seen.[1] In between, and perhaps sticking to, the red corpuscles are seen clear, and sometimes slightly pigmented, rounded, bodies of small size, which in other places may also be seen inside the red corpuscle. To such a clear pigmented, or unpigmented, vacuole-like body the name of *Plasmodium malariæ* is given, and it is considered to be not merely associated with, but the actual cause of the disease.

If these intra-corpuscular bodies are studied (fig. 126) it will be noticed that they enlarge and vary in shape, the outline is irregular, the mass of protoplasm of which they are composed possessing amœboid movements. As the plasmodium gets larger, the pigment also increases in size; and, from being irregularly scattered through the body, becomes regularly arranged round the periphery, and subsequently takes up a central position. The plasmodium now appears to move towards the limiting wall of the corpuscle, and,—either at this stage, or subsequently,—shows signs of division into segments, the various segments meeting by their apices in the dark pigmented centre. A dot of pigment then appears in the centre of each segment. This is known as the **rosette stage.**

The segments then separate from each other; so that in the place of one body we now have several; and, as has been stated, this segmentation may occur inside the red corpuscle, or outside. In the former case, they appear to escape through the corpuscle without rupturing it, and the blood once more contains numbers of very small spore-like bodies, such as have been studied already, and which now undergo the same series of changes as before described.

If the blood be examined freshly, one of the most striking features about such a pigmented plasmodial body present in the red corpuscle is the activity with which the pigment granules appear to move in circles, the planes of which are at right angles to that of the corpuscle in question. This can readily be seen, and is an important point. It serves at once to confute views, which have been expressed, as to the origin of these plasmodia by the breaking-up of the protoplasm of the leucocytes present in the blood.

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[1] See the account given in the *Brit. Med. Journal*, 1893, by Manson, who has arranged the malaria parasites so as to illustrate these various phases of the life-cycle. This coloured plate can now be obtained, mounted on cardboard, from the publishers, Messrs. Bale, Sons, & Danielsson, Ld., Great Titchfield Street, W. Refer also to the beautifully coloured illustrations in Manson's *Manual of Tropical Diseases*, 2nd edition, 1900.

Laveran's crescents.—In certain other forms of the disease, the crescentic bodies of Laveran (*Laveran's crescents*) are seen: each originates as a somewhat oval, vacuole-like, plasmodial body, lying within a red corpuscle, which gradually enlarges and becomes crescent-shaped, the remnant of the original corpuscle subsequently *appearing* to just fill in the space between the two horns of the crescent (see fig. 127, drawn from a specimen kindly given me by Dr. Patrick Manson, C.M.G.)

Flagellate bodies.—If the unprepared blood be examined, preferably in the form of a hanging drop, it will be noticed, after a minimum period of twenty minutes has been allowed to elapse, that besides the above-mentioned appearances, *flagellate bodies* come into view. They are derived mainly from the Laveran's crescents, which straighten out, then swell into elliptical forms, and finally become spheres (p. 244). The pigment is described as occupying a central position at this stage, and suddenly three or four processes, or *flagella*, appear to dart through the limiting wall of the plasmodium. These after some time become detached, and move about independently.

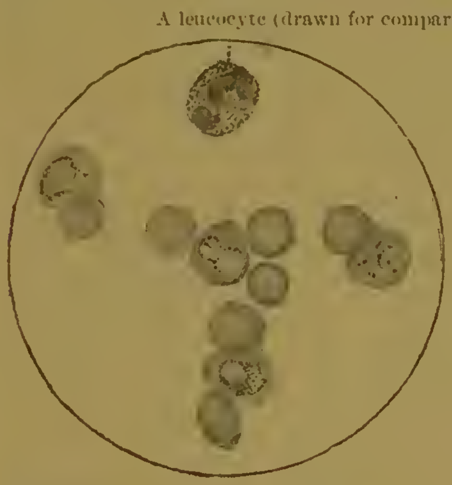

FIG. 126.—PLASMODIUM MALARIÆ WITHIN RED CORPUSCLES. × 530

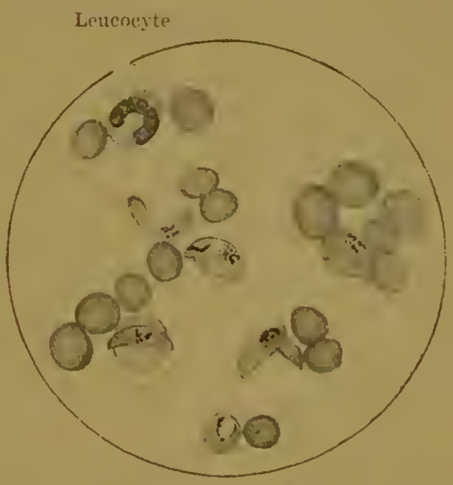

FIG. 127.—LAVERAN'S CRESCENTS AND A SINGLE 'RING FORM' OF PLASMODIUM MALARIÆ WITHIN RED CORPUSCLES. × 530

Flagellate bodies, which are seen in all known varieties of malarial parasites (p. 246), may also arise from forms of the plasmodium, which, instead of passing on to the rosette stage and breaking up into spore forms as usual, pass out of the corpuscle unchanged. The peripherally arranged pigment[1] then becomes deposited more towards the centre; the plasmodial body swells into a sphere, from which flagellate processes in due course appear.

Flagellate bodies are best demonstrated by Manson's method. A blotting-paper cell is first prepared by cutting a slit, a little larger than the film to be made, in a thick piece of blotting-paper the size of a glass slide, upon which it is placed and then moistened. Minute, almost pin-point, drops of malarial blood, known to contain Laveran's crescents in good number, are then collected on another clean slide, previously moistened by breathing on it. By means of a needle, the blood is made into a film about ½ to ¾ inch in circumference, and the slide is at once inverted and pressed down on to the blotting-paper cell. The two glass slides, with intervening blotting-paper, should be kept under a bell-jar. After ½ to ¾ hour the film is dried gently, and fixed in absolute alcohol (five minutes' exposure). The hæmoglobin is washed out by dropping on 15 per cent. acetic acid; the film is well washed in water, and stained for six hours, or longer, in 20 per cent. carbol-fuchsin. It is then washed, dried, and mounted, as usual.

Conveyance of malaria by the agency of mosquitos.[2]—In 1891 Laveran suggested that possibly the mosquito might subserve the malaria parasite much in the same way that it had been shown to subserve *Filaria nocturna*. Manson, in 1894 and 1896, adopted and extended this view, concluding that some extraneous agency —namely, the mosquito—is required to remove the malarial parasite from the blood: that after ingestion the parasite assumes locomotive characters and penetrates the tissues of the mosquito; that it becomes parasitic for a time in this insect; and, just as with the filaria, that only members of one particular species of mosquito (genus *Anopheles*) are effective hosts.

Major Ross, I.M.S., has, by means of numerous observations and experiments, thoroughly established these views: in part, as regards the malaria parasites of man; and completely, as regards one of the malaria-like parasites (proteosoma) of birds. Observations subsequently, in Italy and elsewhere, have served to confirm and extend Ross's work. MacCallum has, amongst British observers, also made important investigations.

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[1] *The pigment*, it should be stated, is derived from the hæmoglobin of the red corpuscles, which accounts for the anæmic and cachectic appearance of patients who have suffered from the disease. After the attack, the pigment becomes deposited in the various organs of the body—the liver, spleen, kidney, &c. *The rosette forms* may not be found in films prepared from the circulating blood, though they may be present, often in large numbers, in the capillaries of the brain and in the spleen (see Malignant Tertian Ague, p. 247).

[2] The following condensed account of recent investigations is taken from the Appendix on 'Malarial Fever,' by Dr. Patrick Manson, vol. viii., Clifford Allbutt's *System of Medicine*. The article should be consulted for details, *e.g.*, as to how the 'travelling vermicule' eventually gives rise to the 'flagellulæ' subsequently mentioned above.

Ross has shown that if blood, from a case of malaria containing the crescent forms of parasite, is ingested by a mosquito, the crescents rapidly become spheres, and many of these then throw out flagella, a process conveniently termed *exflagellation.*

MacCallum has demonstrated the fact that in certain birds another malarial parasite (halteridium) shows similar changes,[1] but the spherical bodies formed from the crescent bodies, as described in the case of man, are of two kinds—one being hyaline, the other granular, and that only the hyaline spheres proceed to exflagellation. They are therefore regarded as representing the male element. The flagella separate from the hyaline spheres, and, by a wriggling eel-like movement, they at once make for the granular spheres or female element, and attempt to bore their way inside. Only one flagellum effects an entrance, and this is followed by a temporary disturbance of the contents of the sphere.

The shape of the sphere subsequently becomes elongated and pointed, or beaked, at one extremity, so as to resemble 'the blade of a broad-headed spear.' The pigment collects at the broader posterior end, and the little body, point first, darts about, and in this way enters a red or white corpuscle. It is spoken of as a *travelling vermicule.* By its powers of penetration it can pass out from the blood ingested into the mosquito's stomach through that viscus, so as eventually to give rise to a wart-like prominence on its outer surface, projecting into the body cavity of the insect. In this stage it is pigmented and soon acquires a capsule.

Spindle-shaped filaments, or flagellulæ, arise in connection with minute spherical bodies forming within the capsules of the numerous wart-like projections. Finally, on the rupture of the capsules, after from six days to three weeks, the flagellulæ are carried by the blood to the veneno-salivary gland in the head of the mosquito. This gland communicates, by means of a long duct, with the base of the middle stylet or lingula of the mosquito, and in this way the flagellula is injected into the tissues of a bitten animal. The flagellula rapidly develops into the fully formed malaria organism, and within a week or ten days of injection the infected animal shows unmistakable evidence of this, its blood now presenting the characteristic parasite.

In considering the mode of infection by malaria parasites the following facts, now fully established, should be borne in mind :—

(i) They are invariably present, in some form or other, in the disease.

(ii) Though they have not been cultivated in the ordinary way, intravenous injection in the blood taken from a patient during an attack of ague produces in a healthy individual a similar attack ; and there is evidence to show that multiplication of the organism occurs in the blood of the person so treated, though mere contact with a diseased patient never brings about the infection.

(iii) A healthy person entering certain regions never previously visited by man may acquire the disease, low-lying marshy districts being well known, especially at certain seasons of the year, to be particularly dangerous. The disease has also been known to arise in large cities in certain parts of the world, when the soil of low-lying, and previously marshy, districts has been disturbed by excavations for building purposes, &c.

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[1] It must be borne in mind, throughout, that the changes described are in the case of malaria blood ingested into the stomach of the mosquito.

Direct infection through the air, and as the result of drinking water in malarial districts, seems undoubtedly to occur, apart from any question of inoculation by the bite of a plasmodium-bearing mosquito.

Varieties of Malaria Parasites

Malaria parasites may be distinguished according to the time required for the formation of spores, and their liberation into the circulation. When the complete cycle takes 24 hours, the parasite, like the type of fever it produces, may be termed *quotidian*. If it requires 48 hours, it is a *tertian*; and if 72 hours, it is a *quartan* parasite.

They may also be arranged according to the type, severe or otherwise, of the fever they produce, quartan and the ordinary form of tertian being classed together as comparatively mild forms, whilst the quotidian and malignant tertian, and other irregular and, generally, more severe, types of fever, may be embraced in a second broad division.[1]

First division.—This includes the so-called *winter-spring parasites*. (i) **Tertian ague** (benign form).—The fever appears at intervals of 48 hours, corresponding with the liberation of a fresh brood of spores into the circulation. The amœboid movements of the parasite within the red corpuscles are very active, more so than in the case of quartan ague. The dots of pigment are very fine, and the flagellate processes are, as a rule, more delicate than in the quartan type. The red corpuscles thus invaded swell up and become paler than those around. When the sporulation stage is reached there is a central spot of pigment, around which a portion of clear protoplasm remains: the peripheral portion of the plasmodium segments up into 15 or 20 rounded spores.

(ii) **Quartan ague.**—There are two entire days between the febrile attacks, i.e. an interval of 72 hours between successive paroxysms; and this again agrees with the time required for the parasite associated with it to pass through its various stages of development. The amœboid movements of the plasmodial bodies within the red corpuscles are not nearly so active as in the tertian type: the pigment granules are also coarser in the quartan variety. Sporulation, which is believed to commence about ten hours before the paroxysm, results in the formation of fewer spores—6 to 12—which contain a central *clear* spot not present in those of the tertian variety.

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[1] Illustrations of these types of parasites, with, and without, attached flagellate processes, are to be seen figured in Muir and Ritchie's *Manual of Bacteriology*, and I have followed the classification adopted by these writers.

The benign tertian and quartan parasites may be as large as, or even slightly larger than, an average-sized red corpuscle.

Quartan parasites may not all mature at the same time. They may develop in two groups, the cycle of the first ending twenty-four hours before that of the second. One clear day, free from fever, intervenes before the next cycle of the first group is completed. This produces the **double quartan** type of fever—two days of fever and one day free. The **triple quartan** type is similarly explained as being due to three sets of parasites completing their cycles on three successive days, the daily liberation of spores giving rise to daily febrile attacks. So for other types of parasites.

Second division.—This includes the more severe and malignant types in which Laveran's crescents occur (*summer-autumn*, or *æstivo-autumnal group* of Marchiafava and Bignami).

(i) **Quotidian ague.**—In the common tropical variety of malarial fever the febrile attack recurs at intervals of 24 hours, corresponding accurately with the time required for the complete maturation cycle of the parasite found associated with it, which is stated to be only about one-third the size of the red corpuscle it has invaded. In this and the next type of fever a quiescent 'ring form' (figs. 126 and 127) is sometimes seen, in which the pigment collects in a circle, being more marked on one side; and there may also be a central ill-defined spot. The spores are very minute, and usually number from 6 to 8, the segmentation occurring almost entirely in the viscera, spleen, kidneys, liver, &c.

An *unpigmented quotidian* is known; as also a fever of *quotidian type* resulting from the presence of *tertian* parasites exhibiting two cycles of maturation separated by an interval of twenty-four hours.

(ii) **Malignant tertian ague.**—The febrile attacks occur at intervals of 48 hours, the time required for the complete development of the parasite, which is very similar in appearance to the quotidian form. Pigmentation has sometimes been noticed to be delayed for the first 24 hours. The amœboid movements of the parasite are very marked; but quiescent ring forms occur in both quotidian and malignant tertian types. Death generally occurs by coma, the capillaries of the brain, as well as those of the spleen, being packed with pigmented parasites at all stages of spore formation, occupying the interior of the red corpuscles.

In both the quotidian and malignant tertian forms the parasite is only about ⅔ the size of the invaded red corpuscle, and sporulation is irregular, no definite rosette, so noticeable in the benign tertian and quartan types, being seen.

There is yet much work to be done in connection with the fevers of irregular type; some of these may be explained, perhaps, on the lines already indicated, as being due to groups of the same parasite maturing at different intervals; or they may be due to a mixture of the different parasites already described; and some may be due to parasites not yet discovered. In this connection a brief account may be given of the disease called 'Blackwater Fever.'

Blackwater Fever, or Hæmoglobinuric Fever

This disease, although specially prevalent in tropical West Africa, and therefore frequently spoken of as 'West African Fever,' is also well known on the Zambesi and in British Central Africa, as well as in tropical parts elsewhere.

It is defined by Crosse[1] as 'a febrile disease of malarial type, characterised by hæmoglobinuria, jaundice, and vomiting.'

Koch considers that blackwater fever itself cannot be malarial, as parasites are absent in more than half the cases; and there may be none of the relapses which are so common in true malaria. He admits that it is pretty frequently associated with malaria, to previous attacks of which, and not to the disease in question, must be attributed the pigment granules found in the various organs.

Crosse suggests, that the condition is one of 'intoxication of malarial origin.' A coloured plate illustrates his paper.

Tsetse Fly Disease

Tsetse fly disease.—It may here be mentioned that recent observation has shown that a somewhat similar hæmatozoon to that causing malaria is found in animals suffering from the tsetse fly disease, which attacks cattle passing through certain belts of country in Africa, so that large expeditions have frequently had to turn back, owing to the difficulty in transport thereby entailed. It has long been known that the sickness is produced as the result of the animals being bitten by the tsetse fly, but its *modus operandi* has only lately been demonstrated. The insect conveys in its body the organisms from previously diseased cattle, and on biting a healthy animal it conveys into its blood the hæmatozoon, and so the disease is inoculated. The animal rapidly sickens and wastes, a fatal result being almost inevitable.

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[1] 'The Histology and Prevention of Blackwater Fever.' *Lancet*, Jan. 9. 1900.

In the blood, when examined, a very motile, elongated, plasmodial body, about double the length of a red corpuscle, is seen, its presence being less readily detected when the motility has ceased. Horses, donkeys, dogs, &c., may be inoculated with the blood, the same symptoms of sickness, wasting, &c., resulting in each case.

Amœbic Dysentery

Amœbic dysentery.—In certain forms of tropical dysentery large quantities of a species of amœba have been found in the contents of the colon, the mucous membrane of which is deeply congested and ulcerated. The amœbæ may penetrate into the walls of the intestine, and, passing through these, reach the neighbouring structures, and so eventually arrive at the liver. In the liver abscesses, which may subsequently follow such cases of dysentery, the pus is frequently found to contain the same species of amœba. It has been noticed that, although the pus first coming from such a liver abscess may contain few or no amœbæ on the first day of examination, the subsequent discharge from the wound may contain large numbers. This has been attributed to the breaking-down of the abscess walls into which these organisms pass.

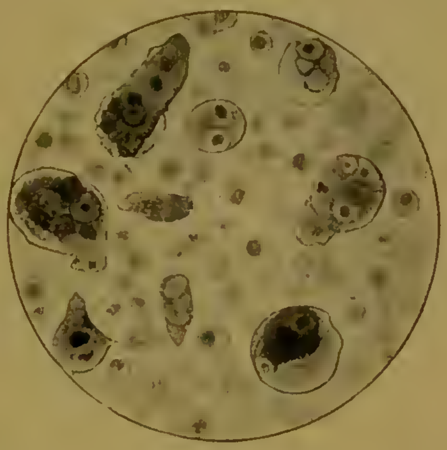

FIG. 128.—AMŒBIC DYSENTERY. × 530

Liver pus, containing the typical amœbæ. Hæmatoxylin and glycerine preparation. (Kindly presented by Mr. Thomas Hart, of the Seaman's Hospital, Greenwich.)

The parasites are readily detected in a hanging-drop preparation. A warm stage should be employed, so that the characteristic, actively motile, amœbæ may be seen darting across the field of the microscope; as, when dead, they are not so readily recognised in unstained pus. They may be seen with a one-sixth objective. They vary greatly in size, many being from four to five times the diameter of a blood corpuscle, and others being considerably larger. They are round or oval in shape, but may be elongated so that their length is from 2 to 2½ times their breadth. The outline is generally fairly well defined, although it may be faint and indistinct. There is generally a central, larger, very granular part containing a nucleus, with nucleoli, and, frequently, vacuoles or clear spaces, containing darker central granules, or food particles. A narrow, clear, peripheral zone surrounds the central granular portion. In the resting condition, a round or slightly oval form is assumed; but, when more active, protoplasmic processes, or pseudopodia, are given off in various directions, and it is by means of these that movement is effected and nutriment absorbed. As will be seen from fig. 128 the body of the amœba often presents the appearance of being folded upon itself. The pseudopodia arise as prolongations, or processes, from the outer, clearer, layer of the amœba; and, subsequently, some of the inner, more central, granular matter flows into each such process.

As has already been mentioned, these amœbæ are found in the intestinal contents of certain cases of dysentery, and preparations made show them enclosed in gelatinous material. Outside the body, the amœbæ and their contents rapidly break up, so that the conditions of their extra-corporeal existence are not yet thoroughly known.

Encysted forms have been described with the capsule having a double contour, the nucleus of which may not always be evident. Sections of the liver beyond the immediate region of broken-down abscess wall, or of the intestine beyond the typical undermined ulcers containing sloughs, show amœbæ in large quantities.

Cultivations have, so far, not been successful for the amœbæ found in dysentery, although the straw infusion at first advocated by Kartulis appears to be a favourable medium for other species.

Rectal injection of intestinal contents from cases of amœbic dysentery has produced in cats and dogs a hæmorrhagic enteritis, in which the amœbæ are present in large quantities, the wall of the intestine, where ulcerated, being invaded with these organisms, but not to the same extent as in the disease when affecting man (Kruse and Pasquale).

To stain coverslip preparations.—The organism may be demonstrated by allowing a drop of hæmatoxylin to diffuse under a coverslip preparation. Glycerine may subsequently be allowed to diffuse under the coverslip, which is then sealed down by painting the edges with melted paraffin.

Another good method is in use at the Seaman's Hospital, Greenwich, and I am indebted to Mr. Thomas Hart for the following details:

Half an inch of pus is placed in a test-tube and covered with about four inches of a solution of hæmatoxylin. The tube is gently shaken, and the pus allowed to settle. The staining fluid is then poured off, and the pus is treated similarly with distilled water, and then with tap water. A little glycerine is now poured down to the bottom of the tube, the stained pus gradually settling down into it. The supernatant watery fluid being removed by a pipette on the following day, film preparations, already stained, can be made from the sediment.

Methylene blue, alone, or combined with eosin, may also be used; and eosin may be combined with the hæmatoxylin method first mentioned.

The amœba coli, in its relation to disease.—Amœbæ have been also found in the normal intestine, in a considerable number of cases; and the relation of these to the amœbæ present in some forms of tropical dysentery is still a matter of doubt. The amœba seen in dysentery differs from the amœba coli proper only in certain small points, it being of greater size and containing coarser granules than the amœba coli, and frequently also containing red corpuscles. It is obvious that the amœba may be merely associated with the disease, and have nothing to do with its causation. But it may be mentioned that amœbæ derived from any non-dysenteric source, when injected into animals, have uniformly failed to produce the positive results such as are reported on p. 250.

There appear to be several different types of dysentery. According to Kruse and Pasquale,[1] they may be arranged under three heads: (1) Amœbic or tropical dysentery, just described; (2) dysentery of the diphtheritic and catarrhal types, in which no amœba can be found, probably due to bacteria of different kinds; and (3) a third form, investigated by Ogata, in which the bacillus is as thin as the *B. tuberculosis*, but much shorter and staining by Gram's method; it is pathogenic to cats and guinea-pigs.

Cantlie, however, divides liver abscesses 'into supra- and intra-hepatic varieties. The intra-hepatic abscess results from dysentery, and the pus contains the usual pyogenic organisms, streptococci, &c., and the amœba coli is perhaps present from the first. The supra-hepatic abscess is non-dysenteric in origin, the pus is sterile, and the amœba coli does not appear in the pus until after the third day of drainage.' (Allchin's *Manual of Medicine*, 1900.)

Psorospermosis, and its Relation to the 'Cancer Parasite.'

Psorospermosis, or Gregarinosis.—**Psorosperms**, or **coccidia**, are oval bodies, belonging to the class of Protozoa (*Gregarinidæ*) found in large numbers in the opaque white patches, of irregular outline, so commonly found in rabbits or guinea-pigs.

From an interesting paper[2] by Sheridan Delépine and Cooper we may quote the following conclusions:—

(1) Oviform psorosperms (*Coccidium oviforme*) are invariably present in the alimentary canal of the rabbit; they are very frequently present in the bile passages

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[1] See Muir and Ritchie's *Manual of Bacteriology*, p. 532.

[2] *Brit. Med. Journal*, October 14, 1891 (sketches and statistics are given).

and gall bladder; and they may produce lesions in the liver, only, in a certain number of cases, varying from 20 to 90 per cent. or more, according to locality, &c.

(2) The prevalence of coccidia and their lesions in the liver seems to be greater in young rabbits, from 600 to 1,200 grammes in weight, than in those younger, or older, than the weights indicated.

Delépine appears to show that a large number of rabbits recover from the invasion of the parasite, a fact supported by the discovery of fibrous nodules, often verified as containing *débris* of psorospermic capsules, in livers otherwise apparently healthy.

(3) The presence of even large quantities of coccidia in the alimentary canal is apparently not incompatible with the perfect health of the rabbit. Psorosperms may, however, undoubtedly bring about fatal lesions.

(4) It is easy to obtain very clear developmental changes in psorosperms allowed to remain 24 to 48 hours in the tissues, after the death of the affected animal.

(5) The authors have not so far observed in any carcinomatous tumour, treated in the same way as psorospermic lesions, any change comparable to those so easily observed in the latter cases; but the presence of psorosperms is not necessarily a sign of serious disease.

Cultivations.—Delépine[1] recommends the study of the development of psorosperms from cultivations made from rabbit droppings in water. In 24 hours, even at an ordinary temperature, a fair proportion of the coccidia exhibit the division of their granular contents into 2, 3, 4, or more parts; and, if kept at the temperature of the body, division occurs even more quickly, and in a large number of psorosperms. In the case of a 'pure cultivation' of this kind made from a liver lesion in water, and not from the intestinal contents (which form a truly luxuriant nidus for development), such rapid division does not occur. Owing to the irregularity of division which may go on, the resulting segments may be round, oval, pear-shaped, equal, or unequal. They exhibit amœboid movements. The ultimate fate of such a divided psorosperm is still a matter of doubt; some segments appear to divide up still further, giving rise to either round, spore-like bodies, or curved rods, termed 'falciform bodies.'

Methods of staining.—Acid fuchsin, or rubin, methylene blue, osmic acid, iodine, and the Ehrlich-Biondi mixture (see p. 257) are all excellent stains, readily demonstrating the parasite at different stages of development from the surrounding tissues. The only part of the nucleus which takes up the colouring matter easily, in hardened specimens, is the nucleolus (Ruffer). Delépine considers that there is a close general resemblance between the so-called 'cell inclusions' of cancer and the psorosperms or coccidia. Every attempt, however, at developing these cancer bodies by the methods which so readily succeeded in the case of the rabbit psorosperm had, so far, only given a negative result (consult, however, p. 257).

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[1] *Loc. cit.*

Paget's Disease of the Nipple

Paget's disease of the nipple.—This disease, which was first described by Paget, and investigated microscopically by Butlin, is one of the greatest interest, as it appears to be a link between the condition known as psorospermosis in rabbits, just described, and cancer proper. It was during the investigation of Paget's disease of the nipple that coccidia-like parasites were first found in the epithelial cells of cancer; and it was consideration of the appearance presented by the epithelium in Paget's disease which suggested closer investigation of the epithelial cells present in cancerous tumours (Watson Cheyne).

Paget's disease simulates an eczema, at first localised to the nipple and areola, whence it may spread over the surrounding skin. Its outline is circular, or somewhat sinuous, the characteristic appearance being a bright red, raw, and somewhat granular surface covered with desquamating scales; in the centre there may be a crater-like ulcer, from which an acrid yellow discharge escapes. This condition may last for years, but in a large number of cases a hard nodule subsequently develops beneath the eczematous-looking patch, which on examination proves to be cancer, for the most part of the spheroidal variety, though duct cancers have been noted in four cases. Butlin[1] showed the primary eczematous condition to be associated 'with a proliferation of the deep layers of the epithelium, with a small cell infiltration of the corium of the areola, dilatation of the galactophorous ducts, and a proliferation of the epithelium, sometimes so extreme as to completely choke them. Deeper down, the epithelium of the acini is seen to be proliferating, and, finally, to be invading the tissues around.'

Darier and Wickham noticed bodies in, and between, the epithelial cells of the skin, which were considered to be coccidia or psorosperms, to which they attributed the disease. Ruffer, Walker, and Plimmer[2] have since shown, however, that these bodies are not parasites, but altered cancer cells, invaginated cells, leucocytes, &c. Whether the condition first noticed in the nipple is really a malignant dermatitis from the first, as has been suggested by Thin, or is a true eczema originally, is a matter of controversy upon which we need not further dwell here.

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[1] Quoted from Erichsen's *Surgery*, vol. ii. p. 801.

[2] *Practitioner*, April 1899.

The Parasite of Cancer

A large series of observations have been made by British and Continental observers with a view of establishing the parasitic nature of cancer, and the appearances seen in the cancerous cells in a large number of cases certainly would suggest the presence of such an organism.

According to Ruffer,[1] Soudakewitch was perhaps the first to clearly recognise the parasite of cancer. Besides a large number of other observers, Ruffer and Walker, Plimmer, Sawtschenko, Foà, Borell, and Galloway have recognised similar bodies as parasites, whereas their existence as such has been strenuously denied by Delépine, Boyce and Giles, and others. Darier and Wickham had earlier described coccidia in cancer; but, as already mentioned, these bodies have since been shown to be cancer cells more or less altered, leucocytes, &c., and they are certainly not parasites. Boyce and Giles[2] are equally positive that the supposed parasites in the cancer cells are merely varieties of endogenous cell-formation. Their paper is illustrated by photographs.

The appearances seen in many cancer cells may now be alluded to. In a very large number of sections, the parasite occupies the cell itself, but in a few of the sections they occur in the nucleus, and may even divide and multiply inside it (Ruffer). This parasite is a small, round, or oval, or sometimes elongated and irregularly outlined cell, varying considerably in size, from ·004 mm. to ·04 mm., or even more, in diameter, with a central portion which stains more darkly than the rest. The dark central point is probably only part of what is, for convenience, termed the 'nucleus,' the rest of which does not take up the stain readily. Around the 'nucleus,' there is a layer of homogeneous protoplasm, staining much less deeply than the nucleus.

The parasite is limited by a well-marked capsule, seen in fresh and fixed specimens, and staining more definitely than either nucleus or protoplasm.

Scattered through the protoplasm of the parasite are often seen small granules of chromatin, and the margin of the cell itself frequently presents a finely striated appearance, which is never seen in the fresh state, and may therefore be due to the fixative. The nucleus often has attached to it a small body with similar reactions, probably a process of reproduction by budding.

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[1] See his well-illustrated paper on 'Protozoa and Cancer' in the *Trans. Path. Soc. Lond.* 1893.

[2] *Trans. Path. Soc. Lond.* 1893.

Staining Reactions

The 'nucleus' of the parasite differs in its micro-chemical reactions from the **nucleus of the cell.** With thionin, a dark purple; with the Ehrlich-Biondi mixture,[1] it stains a copper-red colour; with the hæmatoxylin-fuchsin-orange stain, it also takes a copper-red colour, quite different from the red of either the protoplasm or the fibrous stroma; with the hæmatoxylin-Bordeaux-red stain, it takes a dark claret colour, again darker than that of the protoplasm or stroma.

The protoplasm around the nucleus of the parasite stains as follows: with the Ehrlich-Biondi mixture, reddish-brown, with sublimate as a fixative (or blue, if alcohol is the fixative,[2] staining much less deeply than the nucleus, which is violet, with the same fixative); with hæmatoxylin-fuchsin-orange, the protoplasm and fibrous stroma are red.

The capsule.—With thionin, it is darker than the nucleus (which stains a dark purple), or the protoplasm of the parasite; with the Ehrlich-Biondi mixture, the capsule is of brighter red than the nucleus, or protoplasm; with hæmatoxylin and Bordeaux-red, the capsule appears as a very bright line.

Ruffer and Plimmer[3] describe the multiplication of the parasites as occurring by fission into two, or multiples of two, subdivision of the parasites being followed by division of the capsule; in this way a kind of cyst, or, rather, an agglomeration of parasites, is formed, which may consist of as many as 32 or more young protozoa, each with its own capsule, a process of segmentation resembling that which takes place in the protozoa of malaria.

Plimmer,[4] whose brilliant investigations on cancer have now extended over more than ten years, has recently reported that he has met with the parasitic bodies, described by Ruffer, Walker, and himself, in 1,130 out of a total of 1,278 cases, failing to find them in 63, which were densely fibrous and atrophic—i.e. cured locally; in 27 others, there was so much degeneration that no stain could be made to affect the broken-up and dead cells. The parasites are not found in all parts of the cancer, being only certainly detected at the growing edge, and in active cells, not in those showing retrograde or degenerative changes. They may also be seen in leucocytes, either within the cancer cells, or free.

In very rapidly growing cases, there may be scarcely a cell which does not contain one or more, up to over sixty, of these parasites. These acute cases are very rare, only nine out of the 1,278 examined by Plimmer.

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[1] These methods of staining are mentioned subsequently.

[2] Alcohol is of no use as a fine fixative.

[3] *Trans. Path. Soc. Lond.* 1893.

[4] The 'Special Cancer Number' of the *Practitioner*, April 1899. Plimmer's article in this number is by far the most important recent contribution to the ætiology of this subject.

Examination of fresh specimens.—The parasitic bodies in the cells can be seen and examined without any stain, on making a film preparation from a scraping, made with a very sharp knife from the cancerous growth, with or without the addition of a 0·75 per cent. salt solution. A $\frac{1}{12}$-inch oil-immersion lens must be used, with an Abbé condenser, the iris diaphragm being nearly closed, so as to cut down the cone of light rays as much as possible: or, without a condenser, the concave mirror placed to get as oblique a pencil of rays as possible, may be used.

The best stain to use for fresh specimens is an old solution of Löffler's methylene-blue, or a 1 in 10 solution of polychrome methylene-blue. In the case of stained preparations, the condenser, with plenty of light as usual, is required.

Method of hardening and staining tissues to show the cancer parasite.—Great stress is laid by Ruffer, Walker, and Plimmer on the method of hardening and staining the suspected tissues. Plimmer has lately recommended the two following **fixatives** as giving the best and most accurate results:

1. **Hermann's solution:**

Platinic chloride, 1 per cent.	15 parts
Osmic acid, 2 per cent.	4 ,,
Glacial acetic acid	1 part

The first two ingredients are kept ready mixed, in the dark; but the acetic acid is only added at the time of using.

The quantity of fixative should be at least ten times the bulk of the tissue, which should be as small as possible, *e.g.* about 3 mm.[1]

After 24 to 36 hours in the solution, the pieces ought to be washed in running water for another 24 hours, and then passed through 30, 60, and 90 per cent. and then absolute alcohol, remaining in each for 24 hours. They are passed through cedar-wood oil into paraffin; an alternative method of imbedding being mentioned in Appendix B.

Plimmer says this is the best fixative known to him, giving the best results with both nuclei and protoplasm. But it must be used with care to obtain constant and accurate results.

2. **Perchloride of mercury.**—A solution of boiling 0·75 per cent. salt solution is saturated with corrosive sublimate; when cold, the solution is decanted from the subjacent crystals, and it should be kept in a dark glass bottle. At the moment of use, 5 per cent. of glacial acetic acid is added.

After 24 hours in this solution, Plimmer advises that the small pieces of tissue used should be put directly into 70 per cent. alcohol, to which may be added a few drops of a saturated solution of iodine in spirit.[2]

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[1] Approximately 3 mm. = $\frac{1}{8}$ inch.

[2] *Not* the tincture, or Gram's solution, as is often done; these contain iodide of potassium; and iodine in potassium iodide precipitates perchloride of mercury (Plimmer).

The alcohol is increased in strength daily until absolute alcohol is reached. The tissues are then passed through cedar oil into paraffin.

Corrosive sublimate, though not so good a fixative as Hermann's, can, however, with care be made almost as good, and it is much cheaper and more convenient to use.

The stains[1] recommended by Plimmer, as giving constant and good results, are:

(*a*) **Thionin.**—This is the quickest method, and if carefully done gives good results.

(i) Use a concentrated watery solution, stain for 15 to 30 minutes.

(ii) Dehydrate with acetone-alcohol (acetone 1 part, absolute alcohol 5 parts).

(iii) Transfer to xylol, and mount in balsam.

Either fixative mentioned above can be used, the sublimate often producing a beautifully differential metachromatism.

(*b*) **Double staining,** using Heidenhain's iron hæmatoxylin as a nuclear stain.[2]

(i) Place the sections for 2 hours in a 4 per cent. solution of ferric alum.

(ii) Wash, and place the sections in a ·5 per cent. solution of hæmatoxylin in water for half an hour.

(iii) Treat again with the ferric alum, which decolorises the sections. This part of the process ought to be watched under the microscope, and the decolorisation stopped when the nuclei are darkly stained, and the cytoplasm of the cancer cell is nearly colourless.

(iv) When decolorisation is sufficient, wash the sections in running water for some minutes.

They are then ready for the second stain, which differentiates the parasites. One of two differential stains may be used:

1. A combination of **acid-fuchsin** and **orange G**, originally used by Ehrlich, and made as follows:

Saturated aqueous solution of acid-fuchsin	3 parts
,, ,, ,, orange G	150 ,,

This can be used undiluted, or diluted with 2 to 4 parts of water, and this is advisable when using sublimate as a fixative. With Hermann's fixative, it will require 3 minutes with the strong, and 15 minutes with the weak solution. Using the sublimate fixative, less than 1 minute with the strong, and about 3 minutes with the weak solution should be allowed.

The sections are best cleared in origanum oil, but clove oil can be successfully used with care.

2. A 1 per cent. aqueous solution of **Bordeaux red.** This is an excellent differential, double stain.

If Hermann's fixative has been used, the section is kept in the stain for 10 minutes; in the case of sublimate, allow only 3 minutes. Clear with xylol.

If the sections are over-stained, clear with clove oil.

(*c*) **The Ehrlich-Biondi mixture.**—One of the earliest stains used for demonstrating the cell-inclusions. To use this stain, so as to secure proper differentiation, is not at all easy, and Heidenhain's method[3] must be carefully practised.

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[1] For the various colour effects, respectively produced, see 'Staining Reactions,' p. 253.

[2] Plimmer considers this to be the finest nuclear stain, and it can be used equally well with Hermann's or the sublimate solution fixative. The sections must, however, be very thin; and this is, indeed, essential in all cases; the tissue should also be as fresh as possible, perfectly fixed, and accurately stained.

[3] Consult von Kahlden's *Pathological Histology*, p. 51.

The method is as follows :

(i) The section, fixed in sublimate, is stained with the diluted solution (*i.e.* 1 in 100 of the saturated solution, with acetic acid added, as directed) of 'Biondi'—for 6 to 24 hours.
(ii) Rinse in water.
(iii) Treat with 90 per cent. alcohol for a short time.
(iv) Dehydrate in absolute alcohol ; clear in xylol, and mount in balsam.

Cultivation of the parasites found in cancer has been successfully achieved by Plimmer, using the following **medium.**

An infusion is made from cancer-tissue, just as is done in the case of ordinary beef infusion. To this is added, after careful neutralisation, 2 per cent. of glucose and 1 per cent. of tartaric acid.

Into this, very small pieces of growth, cut out with all possible precautions against contamination, are placed ; and then the flasks and tubes are kept under anaërobic conditions, the air being exhausted and hydrogen substituted for it. Such **anaërobic growths** maintain their virulence for at least 4 months.

After about 48 hours' incubation, a cloudiness of the medium is produced, increasing until about the sixth day, when the growth sinks to the bottom, leaving the liquid clear, no scum or pellicle being formed. On this medium, solidified with agar, form small, separate round colonies, of white colour, changing to yellow in a few weeks.

The organism thus isolated corresponds to the description already given, many young cells having at first no capsule, but containing the central, deeply staining, 'nucleus,' so called.

Inoculation of the growth. Actual new growths have been found by Plimmer after intraperitoneal injection of guinea-pigs, which died in 13 to 20 days, with innumerable, small, transparent nodules studded over the peritoneum, and mottling the liver, spleen, omentum, and intestines. The lungs were full of transparent growths, especially visible on the surface.

Microscopically, these growths are of endothelial origin, the cells contain the organisms, and there are also many normal and degenerate ones in the intercellular tissue ; there is also a great deal of round-celled infiltration in the neighbourhood of the growth.

Inoculated subdurally into rabbits, no true neoplasms were formed, but the organisms were found in the brain, cord, liver, kidney, and blood.

Inoculated into the scarified rabbit's cornea, in 3 to 5 days considerable proliferation of the corneal epithelium was found, together with the organisms in the cells, the proliferation showing itself not only on the surface, but also in the fibrous layers of the corneal tissue, into which masses of enlarged epithelial cells had extended.

Pure cultures can be made from the tumours described, and, when inoculated into suitable animals, they produce again similar growths. Whether they are to be regarded as true cancers is a matter for consideration.

Plimmer has thus clearly demonstrated that the *cell-inclusions are living organisms*, and that they are probably not protozoa, but are related to the saccharomycetes. While making no claim to have discovered *the cause* of cancer, he has produced, experimentally, malignant tumours in animals by inoculating an organism isolated from a malignant tumour in man. His paper, in the 'Practitioner' for April 1899, is beautifully illustrated, and should be carefully studied.

Still more recently, Lambert Lack has published the result of one experiment, in which nodules of cancer-like growths, microscopically, appeared scattered over the peritoneal cavity of a rabbit after inoculation with fresh scrapings from the interior of the split-open normal ovary.

If this observation should be confirmed, the parasitic theory must be taken as one of several possible modes in which cancer may originate.

LESSON XXII

BACTERIOLOGICAL EXAMINATION OF AIR, WATER, SOIL, AND MILK

Air

Air.—I. *Qualitative examination.*—Expose to the air of the room gelatine and agar-agar plates, placed on shelves at different levels, for varying lengths of time (*e.g.* from 1 to 15 minutes). Incubate at the appropriate temperatures for these media, and compare the organisms found under the varying circumstances of growth. Enumerate the colonies appearing on successive days.

II. *Quantitative examination.*—For this purpose several methods may be used, the general principle in all being that a known volume of air is aspirated through the apparatus, and the organisms contained therein sooner or later brought into contact with some culture medium, such as nutrient gelatine. A roll-tube is made, and the colonies are counted in the usual way (pp. 27, 28), waiting for at least three or four days before the final count is made.

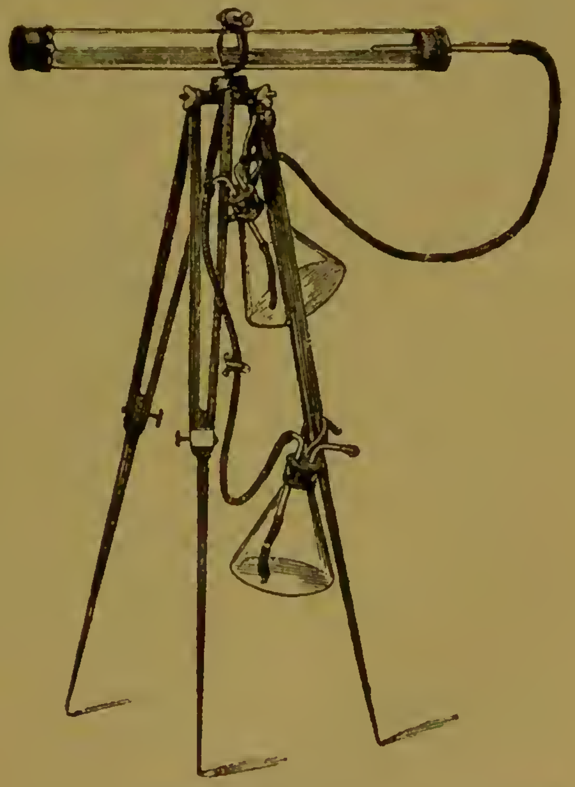

FIG. 129. HESSE'S APPARATUS, ON PORTABLE STAND, FOR THE BACTERIOLOGICAL EXAMINATION OF AIR

The method of using the two Erlenmeyer flasks as an aspirator is explained in the text.

(i) **Hesse's method.**—This method is not much used, in actual practice, now. Hesse's apparatus (fig. 129) consists of a long glass cylinder, 70 × 3·5 cm., closed at one end with an india-rubber plug through which passes a glass tube packed with wool, connected at its outer extremity with a piece of rubber tubing passing to the first of two Erlenmeyer flasks containing water, by manipulation of which aspiration of the air through the cylinder can be produced. The other end of the cylinder is closed with two rubber caps, the inner one having a centrally placed, circular aperture, 10 mm. in diameter.

The glass cylinder, caps, and plugs are sterilised by means of a 5 per cent. solution of carbolic acid, which is then washed away with alcohol. After draining off the alcohol, 50 c.c. of nutrient gelatine are poured into the cylinder. The apparatus is sterilised in the steamer for three successive days. A roll tube is then made in the usual way. The Erlenmeyer flask nearer the glass cylinder is connected with the glass tube passing through the rubber plug, after the wool has been removed from the tube. The apparatus is placed in the air to be examined, and the outer of the two india-rubber caps is removed from the other extremity of the glass cylinder. When the flask nearer the cylinder is tilted, the water it contains flows into the second flask, with which it is connected by a piece of tubing. The water is thus completely siphoned off from the flask, and the air is aspirated into the cylinder to replace it. The position of the flasks is then changed, so that the empty one now occupies the lower position. The siphon action again comes into play, and thus more air is sucked through the cylinder, until the required volume of air has been passed over the roll-tube. When this has been accomplished, the tube is capped at both ends, and the colonies subsequently appearing in the gelatine counted in the usual way.

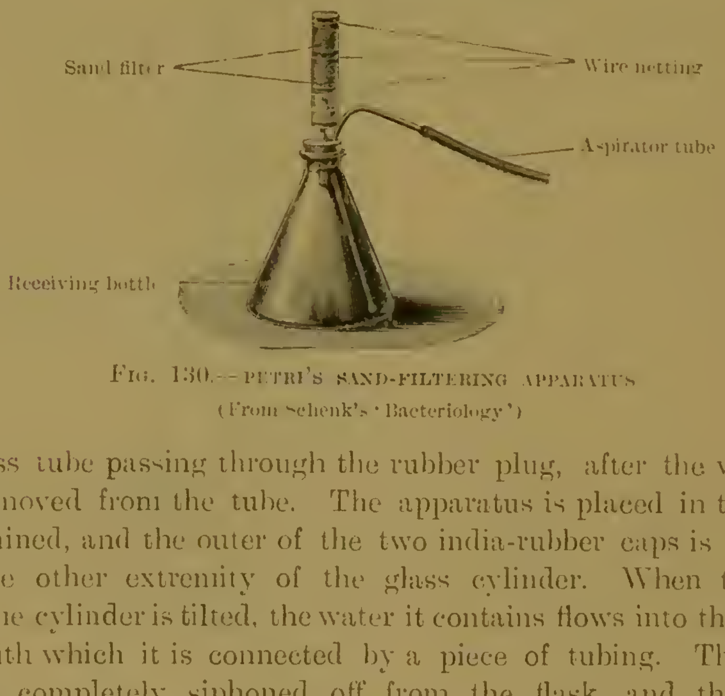

FIG. 130.—PETRI'S SAND-FILTERING APPARATUS
(From Schenk's 'Bacteriology')

Hesse's apparatus, as already mentioned, is not now much used, owing to the greater accuracy and cheapness of more modern methods.

(ii) **Petri's method.**—Petri's apparatus (fig. 130) consists of a vertical glass cylinder, fitting, by means of a small glass tube passed through a rubber plug, into a conical Erlenmeyer flask. The cylinder is packed with sterilised sand, and a known volume of air is drawn through the sand by means of an aspirator, connected with the conical flask by a second, longer piece of glass tubing passing through the rubber plug to the bottom. The sand through which the air has been drawn is mixed with liquefied gelatine, and the colonies developing when the 'plate' is made are counted.

The objection to Petri's apparatus is that fine granules of sand may be mistaken for developing colonies.

Sedgwick-Tucker aërobioscope.—This apparatus enables one to overcome the difficulty just mentioned, the sand being replaced by sterilised sugar, which dissolves in the liquefied gelatine after a known volume of air has been drawn through the apparatus. The aërobioscope consists of a cylindrical glass tube (fig. 131), measuring 15 cm. × 4·5 cm., one extremity of which is constricted so as to form a narrow neck 2½ cm. in length, which is plugged with wool; the other extremity terminates in a fine glass tube 15 cm. × 0·5 cm. The narrow glass tube is plugged with wool, and at a distance of 5 cm. from the plugged extremity a piece of brass wire gauze is inserted so as to support the sterilised granulated sugar, which fills the rest of the tube up to its connection with the cylinder, when the apparatus is placed in a vertical position with the glass tube in the dependent position, during the aspiration of the air.

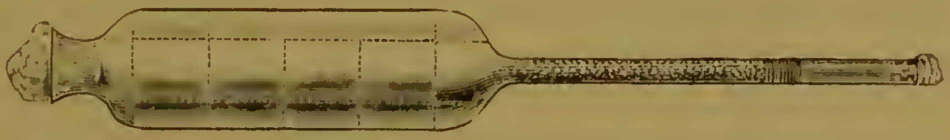

FIG. 131.—AËROBIOSCOPE EMPLOYED IN THE METHOD OF SEDGWICK AND TUCKER, FOR THE BACTERIOLOGICAL ANALYSIS OF AIR

The aërobioscope is first cleaned, and dried, and sterilised in the steamer; the plug is removed from the neck of the cylinder and the sterilised sugar inserted, so as to fill up the glass tube to the extent indicated above, the wool plug being replaced in the neck, and the apparatus is then kept in the hot-air steriliser at 120° C. for three hours. The air may be drawn through the apparatus, after removal of the wool plug, by means of the ordinary graduated aspirating bottles used in the case of Hesse's apparatus (fig. 129), or by means of the water aspirator, in which the weight of water that flows away indicates the volume of air that is sucked through the aërobioscope. Either of these kinds of aspirators is more convenient than the air-pump.

After a known volume of air has been drawn through, the wool plug, freshly sterilised by passing through the flame, is inserted in the neck of the cylinder, which is then placed in a nearly horizontal position. The sugar is tilted from the glass tube into the cylinder, the process being assisted by a series of gentle taps.

By means of the special cylindrical glass funnel with bent stem (fig. 132), 20 c.c. of sterile liquefied gelatine are carefully inserted into the cylinder and the plug of wool replaced. The liquefied gelatine dissolves the sugar. A roll-tube is made in the usual way (pp. 21, 22), solidification being accelerated by the addition of blocks of ice in the water in which the cylinder is rolled. The colonies which develop are counted, as in the case of a roll-tube, the squares which are already marked out in the glass cylinder greatly facilitating this process.

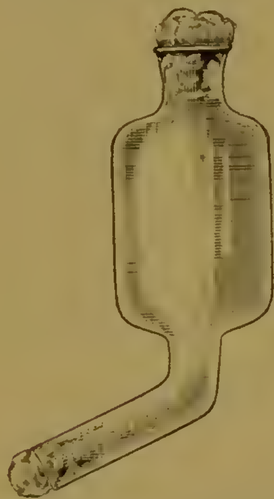

FIG. 132.— CYLINDRICAL GLASS FUNNEL WITH BENT STEM, USED FOR TRANSFERRING LIQUEFIED GELATINE INTO THE AËROBIOSCOPE

Abbott,[1] whose description I have followed in the above account, considers this to be the best method of examining the air, bacteriologically.

For the complete bacteriological investigation of the air, **anaërobic organisms,** blown about in the form of spores derived from the soil, &c., must be sought for, employing for their cultivation the methods already described in the lesson on Anaërobes, modifying them as in the following instance:

A known volume of air is drawn through a wash-bottle containing glucose-gelatine, kept liquefied by placing the flask in a water-bath at 38° C. The inlet tube is then fused, and the flask completely exhausted. The exit tube is then fused, and the rubber cork coated with melted paraffin, as usual, wherever there is any danger of the air entering the apparatus.

If, after incubation at 22° C., colonies appear, they may be counted after a few days, and subcultures, in any of the ways already recommended, may be made to determine their exact nature.

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[1] *Principles of Bacteriology*, pp. 553-56.

Water

Qualitative examination.—This is made by inoculating tubes of gelatine and agar-agar, and plating in the usual way, petri-dishes, of course, being employed instead of the original, very much less convenient glass plates. It is necessary, also, to have the plates diluted in the ordinary way.

Quantitative examination.—For the quantitative examination of water, the greatest care must be taken that the flask used for collecting the sample is sterile, and the cultures made as soon after collection as possible, as delay means the multiplication of organisms. Where the plates cannot be made speedily, the vessels containing the samples should be kept as cool as possible, by surrounding them with ice, or by packing them in some non-conducting material, such as sawdust.

A Winchester quart may be used to collect the water, the bottle having been previously washed out with commercial hydrochloric acid. The acid is removed by thoroughly rinsing in ordinary tap water, and the bottle is then sterilised in a hot-air chamber at 150° C. for three hours, or in a steam steriliser for three successive days, half an hour each day, the bottle being carefully stoppered and covered with sterile wool, which is removed only at the time of obtaining the sample. Generally, it is sufficient to rinse the bottle with water, then with commercial acid, finally well washing out with the water to be collected.

Smaller quantities of water may be more conveniently collected in small glass bulbs, which have been made by fusing the end of a glass tube, and blowing it out into a globe in the usual way. The other extremity is drawn out into a fine capillary tube and sealed until required for use, when its extremity is broken off under water; the vacuum, produced by the cooling of the previously heated globe, causes the water to rush up and partially fill it. The extremity of the tube is then sealed; and, after packing in ice, it is at once forwarded to the laboratory where the examination is to be made. To inspect the water, after nipping off the end of the tube, the bulb is gently warmed—not boiled—over a small gas jet or spirit lamp, care being taken, of course, to avoid sterilising the water in this process. Each bottle, or bulb, should be kept in a canister, which should only be opened at the moment of collecting the sample of water. Great care must be taken not to contaminate the mouth of the bottle, or its stopper, which should be removed with sterile forceps, in which it should be held, and *not put down anywhere*, until finally replaced.

Tap water should be allowed to flow for five minutes before being collected; and water from the mains should always be taken, instead of from a cistern, except for special reasons.

In sampling water from a river, reservoir, &c., the closed bottle should be immersed, so that the mouth is at a distance of 1 foot from the surface before the stopper is removed, and this is then replaced at the same depth before bringing the bottle to the surface, taking care to disturb shallow water as little as possible.

Should a sample be desired, not from the surface but from a given depth below the surface, the apparatus devised by Miquel (fig. 133) may be employed.

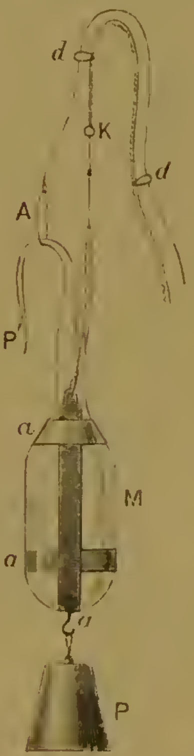

FIG. 133.—MIQUEL'S APPARATUS FOR COLLECTING SAMPLES OF WATER AT DEFINITE DEPTHS

(From Frankland's 'Micro-organisms in Water.')

The following description is given by Frankland in his work on 'Micro-organisms in Water.'

'It consists of a glass vessel M, of about 50 c.c. capacity (the neck of which is drawn out to a fine point and bent, as seen at P'), held in its place by metal bands *a a a*, to which a weight P, of from 2 to 3 kilograms, is attached. The whole is suspended by means of a strong cord graduated in metres, yards, or feet by means of rings and knots. Running alongside of this cord a thread of copper passes through the rings *d d*, situated a metre, or yard, apart. Attached to the end of the copper thread is a ring A, which encloses the fragile neck of the vessel. When the apparatus has been lowered to the required depth, the neck is broken off by giving a quick sharp pull to the copper wire, and the water rushes into the sterilised and vacuous vessel. It is then drawn up, and the depth carefully noted to which it has been lowered. If the water is not to be immediately examined, the broken neck of the vessel must, of course be sealed in the flame.'

Quantitative bacteriological examination of water.—To determine its purity from a bacteriological aspect, known volumes of the water are mixed with gelatine and agar-agar, respectively, and a single plate made in each case, the volume of water employed varying according to its impurity. If, for instance, a preliminary plate made by the addition of 0·5 or 1·0 c.c. of water is found to contain too many organisms for ready enumeration, or if the liquefying organisms present are in great abundance, fresh plates must be made containing a still less quantity of water, *e.g.* 0·1 or 0·25 c.c. Gelatine plates are incubated at 22° C., and those made with agar at 37° C. Colonies are counted daily by means of Wolffhügel's apparatus, or Pakes's enumerating disc (figs. 19, 20, pp. 27, 28), the final count not being made before the fourth day.

It should be noted that the organisms ordinarily found in water develop much more rapidly at the lower temperature of 22° C. on gelatine, than at the higher, 37° C., on agar. However, agar-agar kept at 37° is a more favourable medium for the growth of any pathogenic organisms which may be present in the water. In counting the colonies it should be stated with which medium, gelatine or agar, the plate culture has been made.

Generally speaking, in the case of organisms ordinarily found in water, the proportion of colonies appearing on gelatine plates, compared with those on agar-agar, is stated to be as 2 to 1, or in some cases as 5, or even 10, to 1. But where the water has come from a well, long disused and contaminated with sewage, the numbers of colonies appearing on gelatine and agar plates respectively have been known to be approximately equal.

Special methods for the detection of certain pathogenic organisms.

I. *In the case of spore-bearing bacilli*, such as *B. anthracis*, Frankland recommends the elimination of the non-spore-bearing organisms by raising the temperature of the medium, with which the suspected water is mixed, sufficiently high to kill the bacilli, but leave the spores intact.

Frankland[1] gives the following details of his experiments: From 1 to 3 c.c. of the water containing anthrax spores were mixed with 1 c.c. of sterile broth, and heated for periods of from two to five minutes to 50° C., to 70° C., or to 90° C.; after which they were submitted to ordinary plate cultivation. After heating to 50° C. for five minutes, the 100,000 water bacteria, previously contained in 1 c.c. of the contaminated water, were reduced to from 35 to 39 colonies per c.c., and several of these were recognisable, as colonies of *B. anthracis*. Heating to 70° C. for two minutes reduced the number from 30 to 10 colonies per c.c., in which from 4 to 10 were recognised as those of anthrax bacilli. Heating to 90° C. for two minutes resulted in the appearance of only 7 to 10 colonies, of which 3 to 6 were found to be those of anthrax.

II. For the examination of water suspected to be contaminated by *B. typhosus*, &c., the methods referred to, in the special chapters dealing with those organisms, should be employed.

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[1] *Micro-organisms in Water.*

The purity of water.—Various 'standards of purity' have been proposed, and for an account of these the reader is referred to works on Hygiene.[1] Pure water, according to Miquel's standard, may contain from 100 to 1,000 organisms per c.c., very impure water being defined to contain 100,000, and upwards, per c.c. Filtration to be considered effective, according to Frankland, should remove practically all organisms.

Soil

A loopful of earth from any specific source may be evenly diffused through a tube of liquefied gelatine; and, from this, gelatine plates and roll-tubes of various dilutions made. Subcultures from the different colonies appearing may be made, to isolate the various aërobes, and anaërobes present. Stab-glucose gelatine and glucose-agar cultures should be made. For the isolation of tetanus bacilli, the special method of fractional separation, mentioned on p. 226, may be employed. If necessary, an animal may be inoculated, and the tetanus bacillus isolated from it.

Milk

Milk.—For the examination of milk supposed to be contaminated with organisms, various methods may be adopted, according to the nature of the contagion.

In the case of an epidemic of typhoid fever, supposed to be due to infection of the milk, plates of gelatine, potato-gelatine, and agar-agar may be made in the usual way; and a quantitative examination may be similarly carried out, using 0·1, 0·25, 0·5 c.c. respectively of milk for separate plates.

In the case of tuberculosis, it is best to centrifugalise the milk. The supernatant fluid whey being poured off, the thicker deposit is made into film preparations and examined for **B. tuberculosis,** by the usual methods, Ziehl-Neelsen's being the best. After fixing, and before proceeding to stain the film, it is sometimes useful to clarify it by means of ether, which removes excessive fat.

In the case of diphtheria, the Klebs-Löffler bacilli may be found on centrifugalising the milk and examining the deposit at the bottom of the tube.

In this connection, it may be useful to note that *epidemics of acute*

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[1] For instance, Kenwood's *Public Health Laboratory Work*, p. 432

follicular tonsillitis sometimes occur, apparently dependent on the presence of large numbers of strepto- and staphylo-cocci in the milk supply. This condition appears at times to have become almost endemic when once it has arisen in a public institution. Bacteriological investigation, after centrifugalising the milk, may point to the source of infection, and at the same time serve to distinguish between a simple tonsillitis and true diphtheria.

It should be noted that the **B. coli communis** is commonly found in ordinary milk, where the cows are not carefully groomed every day.

A variety of **Proteus mirabilis** is also not uncommonly found, the very long involution forms,—sinuously curved filaments, with spindle-shaped swellings along their course, or at their extremities,—being characteristic.

LESSON XXIII

METHODS OF TESTING ANTISEPTICS AND DISINFECTANTS

I. Take four tubes containing 10 c.c. of nutrient broth, *a*, *b*, *c*, *d*.

(i) Inoculate (*a*) with a loopful of *B. prodigiosus*. (ii) Inoculate (*b*) with a loopful of *B. typhosus*	(*a*) and (*b*) are to be incubated and used as *controls*, for comparison with (*c*) and (*d*).
(iii) Add $\frac{1}{10}$ c.c. of a 5 per cent. solution of carbolic acid to (*c*). (iv) Add $\frac{1}{5}$ c.c. of a 5 per cent. solution of carbolic acid to (*d*).	Inoculate (*c*) and (*d*) with a loopful each of *B. prodigiosus* and *B. typhosus*. Incubate at 37° C.

On comparing these tubes, after a few days' incubation, (*a*) and (*b*) will show vigorous growths of *B. prodigiosus* and *B. typhosus* respectively; in (*c*), turbidity due to *B. typhosus* only will be seen; *B. prodigiosus* having succumbed to the antiseptic; whilst in (*d*), no growth of either organism will be visible.

II. (i) Prepare *anthrax silk threads*[1] as follows :—

Silk threads, one inch long, are sterilised in the steamer, and dipped for an hour in broth, or normal saline solution, in which has been suspended a loopful of a virulent spore-bearing agar-agar, or potato, culture of anthrax. Using every precaution against sepsis, by means of forceps the threads are transferred in a small sterile petri-dish to the warm incubator, or to the sulphuric acid desiccator.

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[1] These should be previously prepared for the class.

(ii) Transfer some of these prepared silk threads to the antiseptic solutions to be tested, e.g.
  (*a*) Carbolic acid, 1 in 20.
  (*b*) Corrosive sublimate, 1 in 1,000.
  (*c*) Biniodide of mercury, 1 in 2,000.

(iii) With sterile forceps, rapidly transfer a thread from the selected antiseptic solution to sterilised water, and wash well.

(iv) Transfer to an already prepared glycerine-agar plate. Incubate at 37° C. Compare the plates corresponding to (*a*) (*b*) and (*c*), with (*d*), a control plate made by transferring an anthrax silk thread, simply washed well in sterilised water but not exposed to any antiseptic solution.

Comparison will show, what is now a well-established fact, that the biniodide of mercury is a more effective antiseptic than corrosive sublimate solution of corresponding strength.

This experiment, which we owe to **Koch**, may be varied indefinitely as to the variety and strength of germicidal solution employed, and the lengths of time to which the anthrax threads are exposed to them. It is absolutely necessary that the threads should be completely freed from the antiseptic, otherwise the non-appearance of growth on the media may be due to the transference to the media of a minute quantity of the solution, which inhibits the growth, but does not kill it outright.

Herein lies the great practical difficulty in applying this method of testing antiseptics. The salts, especially in the case of corrosive sublimate, often form a firm union with the tissues of the threads, and this holds good even in the case of catgut, which, after soaking in distilled water, renewed day after day for five weeks, still gave characteristic tests for mercuric chloride (Braatz, quoted by Abbott). The addition of ammonium sulphide to the sterilised water in which the threads are washed, before introducing them into the fresh culture media, undoubtedly helps to overcome this difficulty.

On exposing organisms to test the effect of germicidal solutions, it is most essential that, as Abbott points out, the bacilli shall be very finely distributed, so that as far as possible each individual comes into contact with the disinfectant solution.

It seems hardly necessary at this stage to emphasise the fact that, whilst non-spore-bearing organisms as a rule possess but little power of resistance to destructive agencies—such as drying at a comparatively low temperature, heat (dry and moist), and chemical antiseptics, such as carbolic acid (1 in 20), and corrosive sublimate solution (1 in 1,000)—the spores of spore-bearing organisms such as those of tetanus and anthrax maintain their vitality after remaining dry for many months, and sometimes after heating even to a comparatively high temperature, provided this is only maintained for a few moments, moist heat being more destructive than dry.

In **Sternberg's method**, several broth cultures, *e.g.*, of *B. typhosus*, are incubated for twenty-four hours; of these all but one, kept as *a control*, are treated with various strengths of the antiseptic, and incubated for varying lengths of time, *e.g.* for an hour or two, the control also being incubated.

All these tubes are now subcultured; the subcultures are incubated and the effects noted daily.

III. **Disinfection with gases and vapours,** *e.g.* **Formalin, Sulphur dioxide, Chlorine, &c.**

Formalin.—Commercial formalin is a 40 per cent. solution of formic aldehyde, or formaldehyde.

To test the germicidal action of its vapour, solutions of varying strengths from 0·1, 0·5, and upwards, should be employed as follows:—

The apparatus required simply consists of (*a*) a flask, to contain the formalin solution, fitted with a rubber stopper, through which a piece of glass tubing passes for the exit of the vapour; (*b*) a wash-bottle, also fitted with stopper and glass tubes, the water in this flask serving to absorb the irritating vapour after it has passed over the piece of linen rag which has been streaked with, or soaked in, the culture, say, of the Klebs-Löffler bacillus.

The infected linen is inserted into a piece of tubing of rather wider bore than those used for the flasks.

The exit tube from the formalin-containing flask is connected by rubber to one end of this wider tubing, which in turn is connected up to the wash-bottle. On gently warming the flask containing the antiseptic, formic aldehyde vapour is driven off, and so comes in contact with the infected rag in the piece of tubing.

The strength of the solution and the time of exposure to the vapour should be noted. The rag is then quickly dropped into a tube of suitable medium, and the effect noted after several days' incubation.

For the disinfection of rooms, by a modification of this experiment, suitable lamps and tabloids, containing formalin of the required strength, can be obtained.[1] It is necessary, of course, to seal up all apertures, and paste paper over cracks in window-frames, &c., through which the formic aldehyde vapour might escape from the room.

The disinfectant properties of such gases as **Sulphur dioxide** (generated by heating metallic copper with sulphuric acid), and **Chlorine** (obtained from sodium chloride, manganese dioxide, sulphuric acid, and water), can be similarly tested, the gases being washed [2] before they pass over the infected rag.

Such experiments should be carried out in a fume cupboard, owing to the irritating nature of the gases in question.

[1] Experiments carried out by Professor Kenwood and others (*Transactions of Sanitary Institute*, 1897) show that formic aldehyde vapour, obtained by heating formalin, is an extremely convenient and satisfactory agent for the disinfection of rooms. It has great penetrating power, and its germicidal properties, even when much diluted, are remarkable. Owing to the very small percentage of formic aldehyde vapour necessary for the disinfection of rooms (calculated by Kenwood to be approximately only 1 per cent. of the cubic space of the room, *i.e.* of the air in it) this method is inexpensive.

[2] For details, consult Kanthack and Drysdale's *Practical Bacteriology*, p. 131.

APPENDIX A

The Requisites for a good Bacteriological Microscope

ALTHOUGH a very simple and cheap instrument may be all that is absolutely necessary, the student will find it to be truer economy, in the end, to deal only with firms of established reputation, and be prepared to expend from 15*l.* to 17*l.* 10*s.* For the latter sum Messrs. Swift & Son (81 Tottenham Court Road, London), for instance, supply a microscope with all the accessories usually required for bacteriological work.

Besides a rack and pinion coarse adjustment, a *vulcanite* stage (which prevents too rapid cooling during the examination of a hanging-drop preparation, amœbic pus, &c.), a plane and a concave mirror, objectives, and eyepieces, the following accessories should be obtained :

An Abbé condenser, and a mechanical stage, allowing free movement of the slide from before back and *vice versa*, and from side to side.

Swift's mechanical stage, costing 2*l.* 10*s.* out of the 17*l.* 10*s.* above quoted, is not essential, but is a very great convenience, so that it becomes almost indispensable in prolonged bacteriological work.

The vulcanite stage recommended has a ruled scale at one corner, by means of which it is possible always to locate a single organism in a preparation, when once the position of one angle of the slide has been noted and recorded.

The objectives required for ordinary work are the $\frac{2}{3}$ in., $\frac{1}{6}$ in., and $\frac{1}{12}$ in. oil-immersion lens (see p. 29).

The eye-pieces most often used are No. 2 and No. 4.

In selecting objectives and eye-pieces, it must be remembered that clear definition is of more importance than magnification, and that with the higher powers, generally speaking, increase in magnification is associated with more or less loss of definition. Besides clear definition, and freedom from coloured images (due to chromatic aberration), 'flatness' of the field is the great criterion of a good lens. When looking at the centre of the field, the periphery should be almost equally clearly seen at the same time, without altering the focus. This is a point of great importance when choosing the higher objectives, such as the $\frac{1}{12}$ in. oil-immersion lens.

The magnifying power of the objectives made by Messrs. Swift & Son is indicated in the subjoined table supplied by that firm. Thus, with a No. 2 eye-piece and a $\frac{1}{12}$ in. oil-immersion lens, the magnification amounts to 530 times the actual size of the object examined.

TABLE SHOWING THE MAGNIFYING POWER OF OBJECTIVES WITH THE EYE-PIECES MENTIONED THEREIN AT THE TWO POSITIONS OF THE DRAW TUBE NAMED IN TABLE.

Objectives—Draw Tube closed

Eye-pieces	No. 1	No. 2	No. 3	No. 4	No. 5	
1-in.	25	35·5	47	60	77	Diameters
$\frac{2}{3}$	37	50	70	90	115	
$\frac{1}{2}$	60	85	115	145	185	
$\frac{1}{10}$	80	105	150	180	240	
$\frac{1}{4}$	112	168	224	280	335	
$\frac{1}{6}$	175	240	325	420	540	
$\frac{1}{8}$	198	292	386	492	625	
$\frac{1}{10}$	275	412	550	687	824	
$\frac{1}{12}$	385	530	730	925	1180	

Draw Tube fully extended

Eye-pieces	No. 1	No. 2	No. 3	No. 4	No. 5	
1-in.	45	67·5	90	112	140	Diameters
$\frac{2}{3}$	64	96	128	160	192	
$\frac{1}{2}$	107	158·5	214	267·5	310	
$\frac{1}{10}$	126	189	252	315	370	
$\frac{1}{4}$	210	315	420	570	600	
$\frac{1}{6}$	256	384	512	640	690	
$\frac{1}{8}$	342	513	684	855	900	
$\frac{1}{10}$	475	712	950	1225	1270	
$\frac{1}{12}$	550	825	1100	1375	1627	

‘In using the microscope with all powers, from $\frac{1}{4}$ in. objective and upwards, the concave mirror must be used and moved up the pillar on which it slides, until it is stopped by the check screw. The mirror in this position will give the greatest amount of light. With the $\frac{1}{6}$ in. and lower power objectives the flat mirror should be used, no particular distance being essential. These remarks only apply when no sub-stage condenser is used.’

APPENDIX B

Methods for Fixing, Hardening, and Imbedding Tissues

Fixation.—Prior to hardening the tissues, a **fixative** is often desirable, a saturated solution of **Corrosive Sublimate** being the best for general use. (Refer to p. 256.)

Hermann's solution, for cancer parasite work, is also mentioned on the same page.

Foà's solution is also excellent, and is made by saturating Müller's fluid with corrosive sublimate.

How to harden fresh tissues :

i. *Formalin method.*—Small pieces of tissue, not more than ¼ in. in thickness, are placed in a 1 in 4 solution of formalin for fifteen to twenty hours, and then soaked in a solution of gum mucilage, made from gum arabic, for three or four hours. Sections are cut with a freezing microtome and gently brushed off the razor, or planing iron, into distilled water.

[More rapid sections, sufficiently satisfactory for diagnosis, can be obtained, in an emergency, by hardening thin pieces in pure formalin (a 40 per cent. solution of formic aldehyde) for half an hour, and then soaking in gum for another half-hour.]

For the methods of staining, see p. 97 *et seq.*

ii. *Alcohol method.*—To harden the fresh tissue, a small piece is placed for twenty-four hours in each of the following solutions of alcohol:—30 per cent., 60 per cent., 90 per cent., and finally in absolute alcohol. It is then ready for imbedding in paraffin or celloidin, as directed below.

If the freezing microtome is to be used, the tissue need only be passed through 30 per cent., and 60 per cent., of alcohol, and then methylated spirit, avoiding the use of absolute alcohol. Before putting into gum, however, the tissue must be transferred from the alcohol into water, and allowed to soak for an hour or so, to remove the excess of alcohol. This is not necessary when formalin is used.

How to imbed hardened tissues.—A. To imbed in paraffin :

1. From absolute alcohol, transfer to a mixture of equal parts of absolute alcohol and chloroform, till the tissue sinks.
2. Transfer to chloroform for one hour.

3. Then place in a mixture of equal parts of chloroform and paraffin (melting point, 53° C.) in stoppered, wide-mouthed bottles, in which it should be kept for four hours, at 56° C., in the paraffin cupboard.

4. Transfer to pure paraffin for one hour, and then place in fresh paraffin overnight. Keep in the paraffin cupboard at 56° C.

5. Smear vaseline lightly on the inner surface of any convenient little receptacle, cardboard pill-box, coverslip-box, &c. Pour in a little melted paraffin; as it begins to show signs of cooling, transfer to it, with fine forceps, the piece of tissue to be imbedded; cover it with melted paraffin, and allow this to solidify.

Sections are then cut and stained according to the directions given on pp. 99–100.

B. **To imbed in celloidin.**—Thin and thick syrupy solutions of celloidin are made in a mixture of equal parts of absolute alcohol and ether.

The tissue is hardened in alcohol, in the way described. It is transferred from absolute alcohol to a mixture of equal parts of absolute alcohol and ether, for twenty-four hours. It is then kept for twenty-four hours, first in the thin, and then in the thick, solution of celloidin.

Finally, the tissue is placed on a small cube of wood, to which it soon becomes firmly adherent, especially on transferring it to a bottle containing 60 per cent. alcohol. It is then ready for use, and can be kept for any length of time in this solution till required. The cube of wood is then clamped in a Schanze's, or other form of, microtome adapted for cutting tissues imbedded in celloidin. (For further details, see p. 100.)

APPENDIX C

The following tables may be of use to the student for reference and recapitulation of facts, most of which have been already mentioned in the text :

TABLE I

	Gelatine (Liquefied, L., non-liquefied, N.-L.)	**Motility** (Motile, M., non-motile, N.-M.)	**Spore formation** (Present, S., not known, N.S.)
B. prodigiosus	L.	N.-M.	N.S.
B. cyanogenus	N.-L.	M.	S.
B. pyocyaneus	L.	M.	S. (?)
B. fluorescens liquefaciens	L.	M.	N.S.
B. fluorescens non-liquefaciens	N.-L.	N.-M.	N.S.
Micrococcus agilis	L.	M.	N.S.
Sarcina lutea [1]	L.	N.-M.	N.S.
Spirillum rubrum	N.-L.	M.	N.S.
B. subtilis	L.	M.	S.
B. megaterium	L.	M.	S.
B. mycoides	L.	M.	S.
Saccharomyces cerevisiæ	N.-L.	N.-M.	S.
Torula rosea			—
Torula nigra			—
Saccharomyces (Oïdium) albicans			S.
Penicillium glaucum	L.	N.-M.	N.S.
Aspergillus niger			
Mucor mucedo			
Microsporon Audouïni			
Trichophyton tonsurans			
Achorion Schönleini			
Proteus vulgaris	L.	M.	N S
" *Zenkeri*			
" *mirabilis*			

---

[1] *Sarcina pulmonum* and *Sarcina ventriculi*, found in phthisical sputum, and the stomach, respectively, do not liquefy gelatine.

	Gelatine (Liquefied, L., non-liquefied, N.-L.)	**Motility** (Motile, M., non-motile, N.-M.)	**Spore formation** (Present, S., not known, N.S.)
Staphylococcus pyogenes aureus	L. (one var. N.-L.)	N.-M.	N.S.
,, ,, *citreus*	L		
,, ,, *albus*	L. (one var. N.-L.)		
,, *epidermidis albus*	L		
,, *cereus albus*	N.-L.	N.-M.	N.S.
,, ,, *flavus*			
Streptococcus pyogenes	N.-L.	N.-M.	N.S.
,, *erysipelatis*			
Pneumococcus of Talamon-Fränkel	(no growth)	N.-M.	N.S.
Pneumobacillus of Friedländer	N.-L.	N.-M.	N.S.
Gonococcus of Neisser	(no growth)	M. (slightly)	N.S.
Micrococcus tetragonus	N.-L.	N.-M.	N.S.
,, *Melitensis*	N.-L.	M.	
B. aërogenes capsulatus	L.	N.-M.	N.S.
B. anthracis	L.	N.-M.	S.
B. tuberculosis	N.-L.	N.-M.	S. (highly probable)
B. lepræ	(not yet cultivated)		
B. mallei (glanders)	N.-L.	N.-M.[1] (probably)	N.S.
B. diphtheriæ	N.-L.	N.-M.	N.S.
B. typhosus	N.-L.	M.	N.S.
B. coli communis			
B. enteritidis of Gärtner			
Vibrio choleræ Asiaticæ	L.	M.	N.S.
,, *Metchnikovi*			
,, *Finkler-Prior*			
Streptothrix of Actinomycosis	L.	N.-M.	N.S.
,, *of Madura disease*	N.-L.	N.-M.	N.S.
B. of Mouse septicæmia	N.-L.	M. (?)	N.S.
,, *Influenza*	(no growth)	N.-M.	N.S.
,, *Bubonic plague*	N.-L.	N.-M.	N.S.
,, *Yellow fever*	N.-L.	N.-M.	N.S.
B. of Tetanus	L.	M.	S.
B. of Malignant œdema			
B. of Quarter-evil			

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[1] There is, however, marked Brownian movement visible in a hanging-drop preparation (Hewlett).

TABLE II

A table showing the effect of treating certain of the pathogenic organisms by Gram's method.

	Gram's Method of Staining	
	Stained	*Decolorised*
Cocci	*Staphylococcus* (all varieties) *Streptococcus pyogenes*, and *erysipelatis* *Micrococcus tetragonus* *Pneumococcus of Talamon-Fränkel*	*Gonococcus of Neisser* *Diplococcus intracellularis meningitidis* (Weichselbaum) *Diplococcus of non-tuberculous, posterior meningitis* (Still). *Micrococcus Melitensis.*
Bacilli	*B. aërogenes capsulatus* ,, *anthracis* ,, *tuberculosis* ,, *lepræ* ,, *diphtheriæ (Klebs-Löffler)* ,, *murisepticus (Mouse-septicæmia)* ,, *icteroides (Yellow fever)* *Bacillus of Tetanus*	*Pneumobacillus of Friedländer* *B. mallei (glanders bacillus)* ,, *typhosus* ,, *coli communis* ,, *enteritidis of Gärtner* *Vibrio choleræ Asiaticæ* ,, *Metchnikovi* ,, *Finkler-Prior* *B. influenzæ*
	Streptothrix of Actinomycosis ,, *Madura disease*	,, *pestis (Bubonic plague)* ,, *of Malignant œdema* ,, *of Symptomatic anthrax*

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[N.B.—*e* and *f.n.*, at the end of a page-number, indicate respectively *exercise*, or *lesson*, and *foot-note*]

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